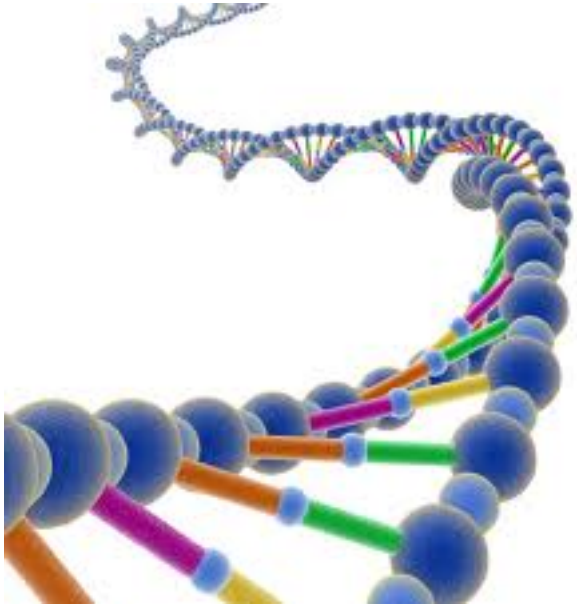
Základy molekulární biofyziky (in English)

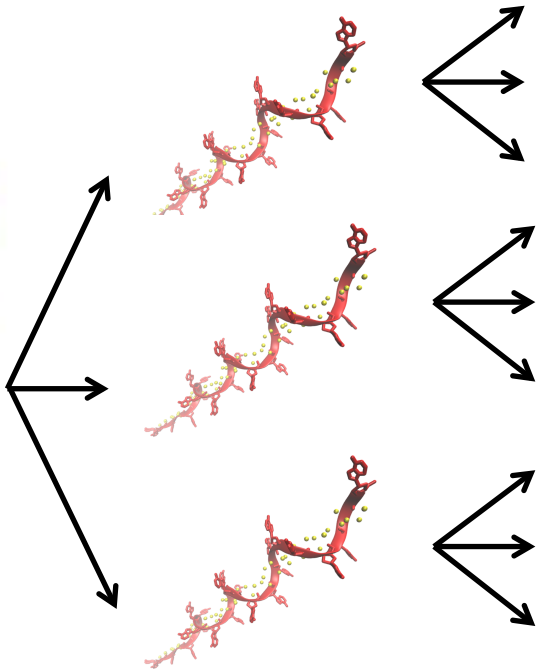
Part 6:
Cellular Structural Biology - NA

DNA as a drug target

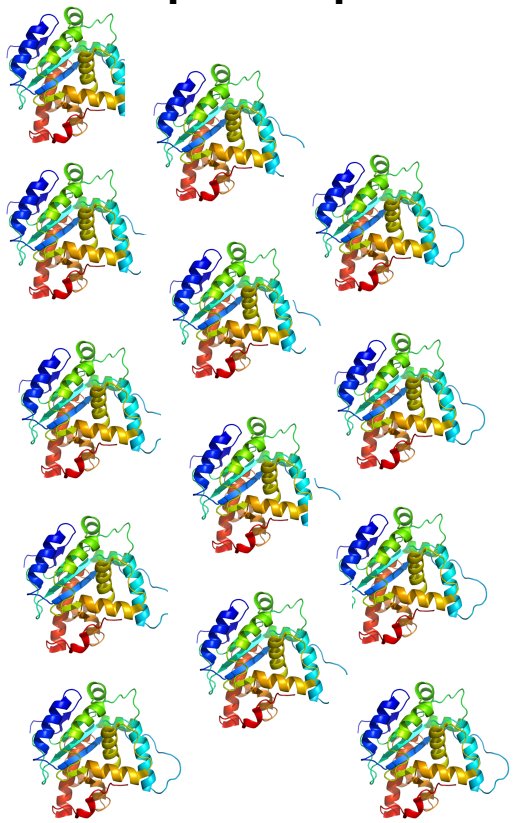
Single gene



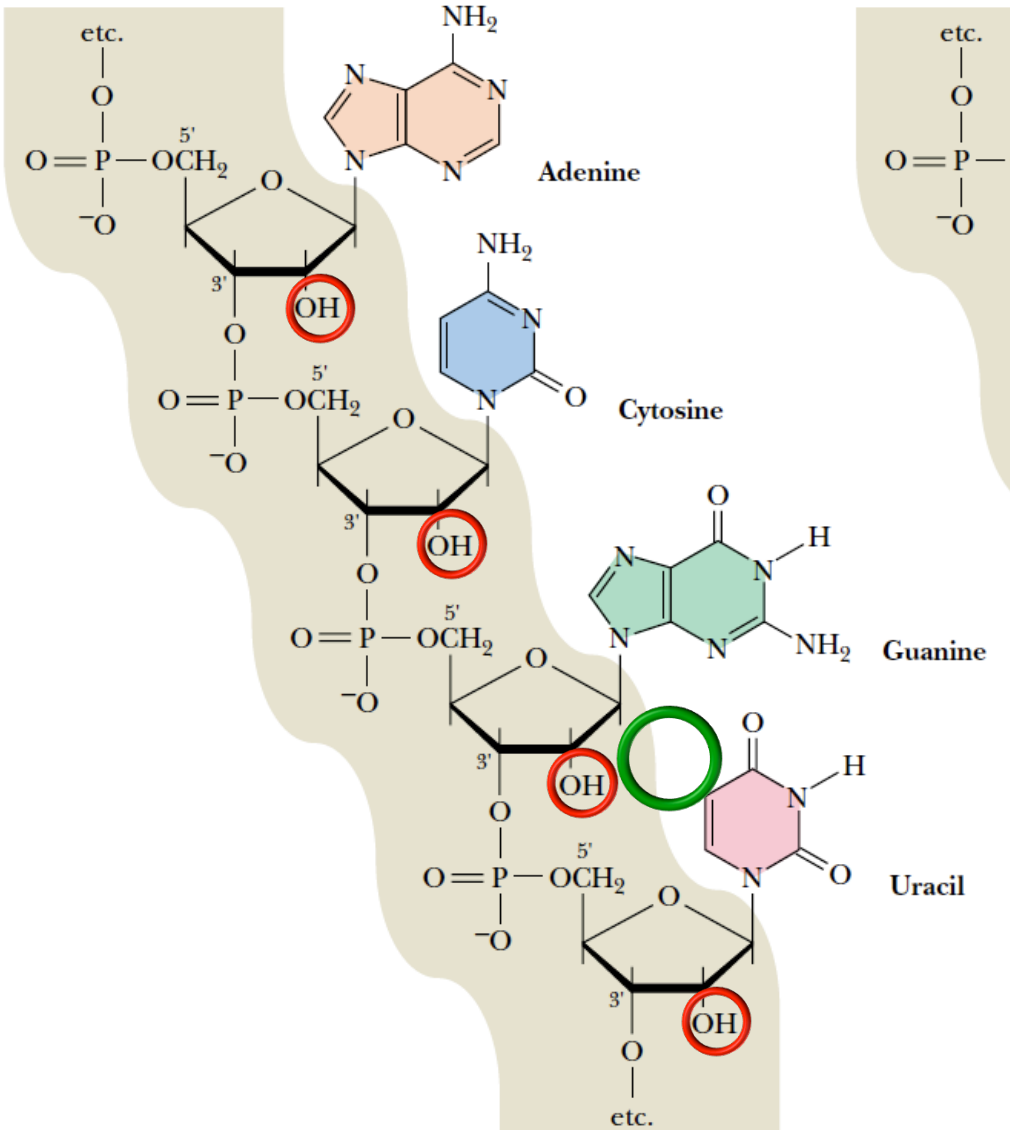
Multiple copies of mRNA



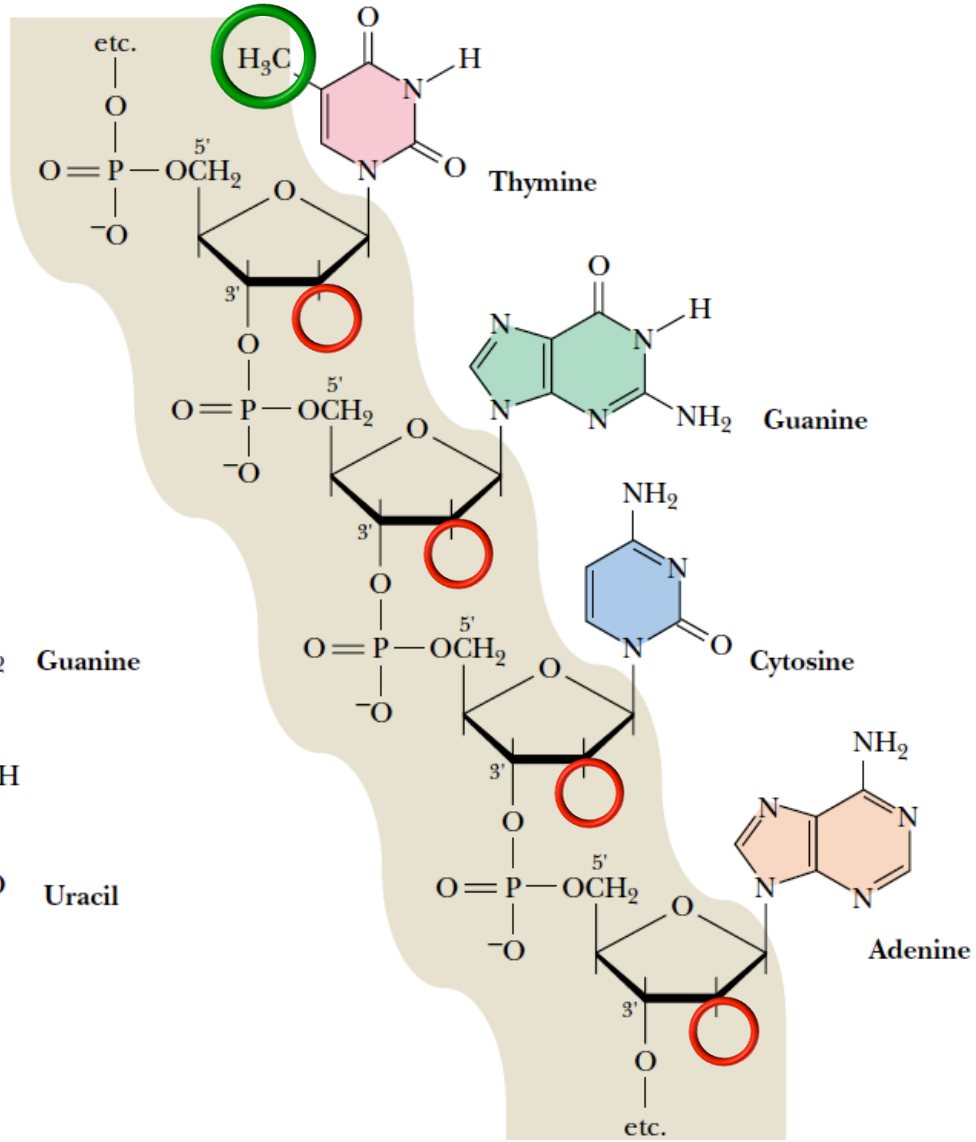
Multiple x Multiple copies of protein



RNA


etc.
Adenine
Cytosine
Guanine
Uracil
etc.

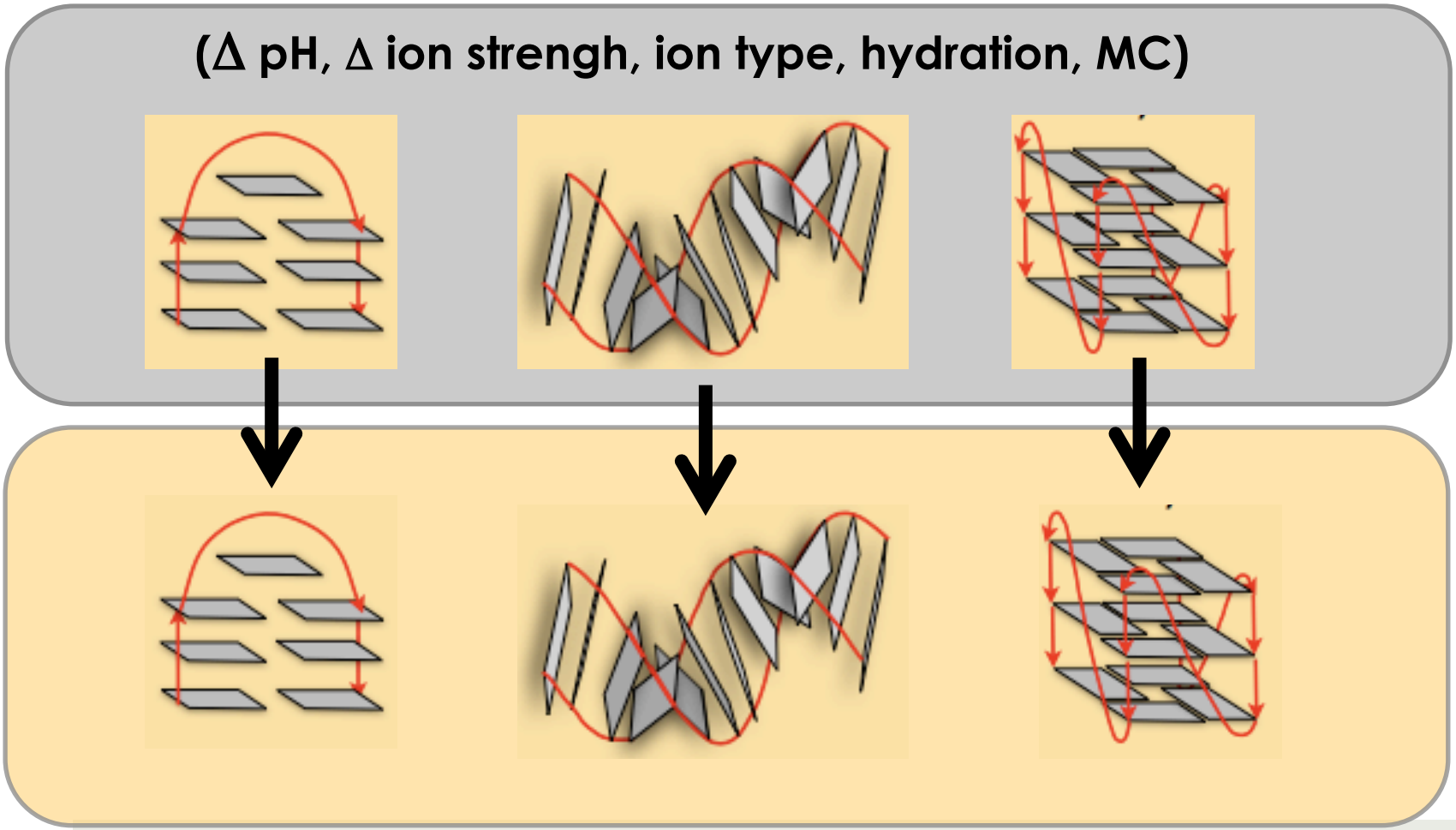
DNA


etc.
Thymine
Guanine
Cytosine
Adenine
etc.

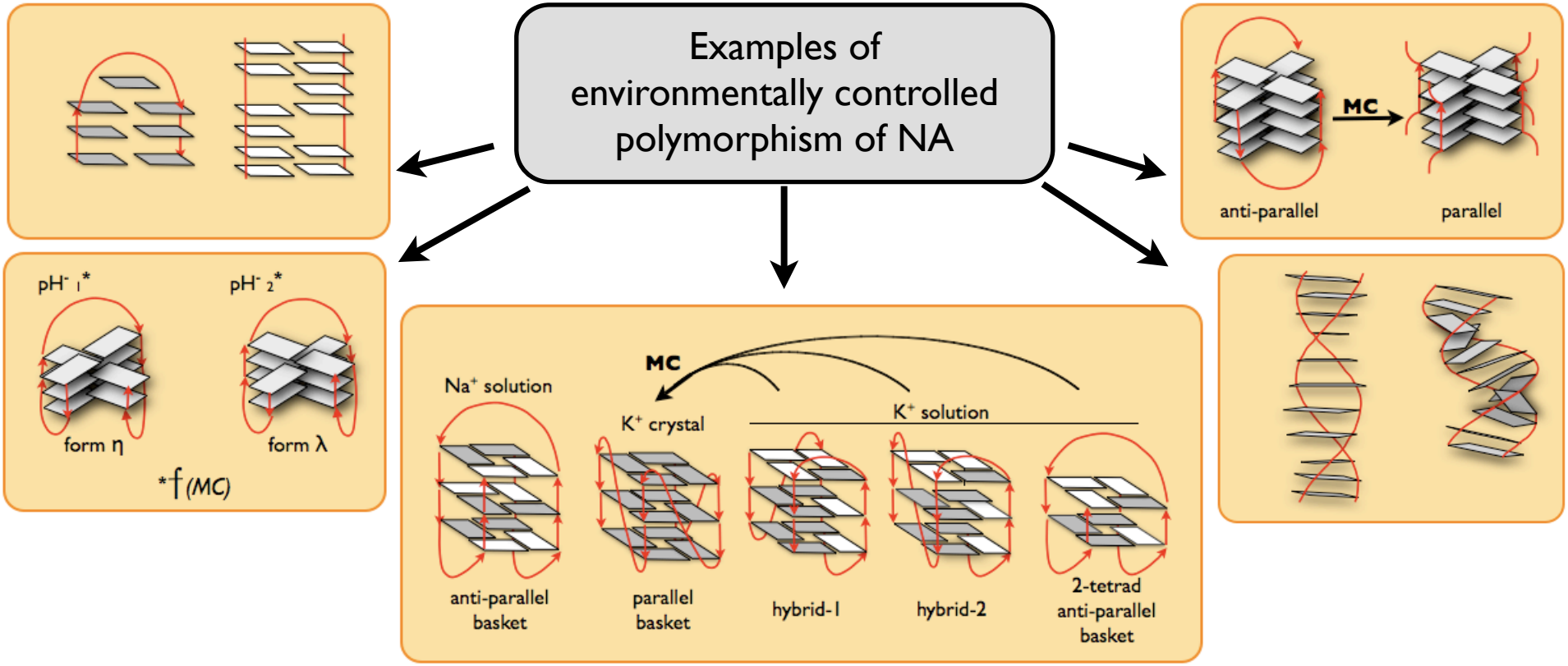
Environmentally Promoted Deformability:

a fundamental difference between DNA and RNA

RNA structure is insensitive to environmental conditions

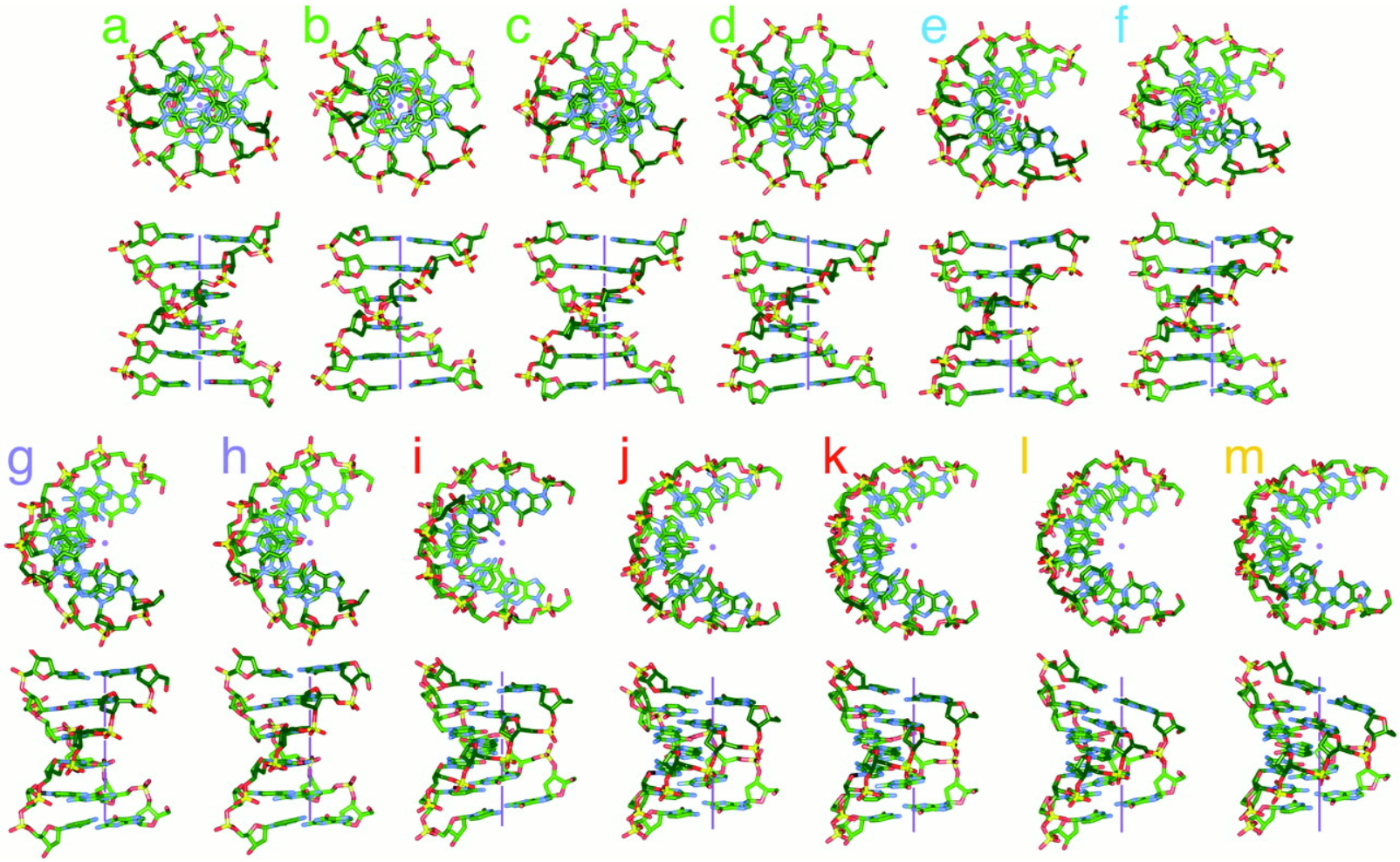


DNA structure is sensitive to environmental conditions



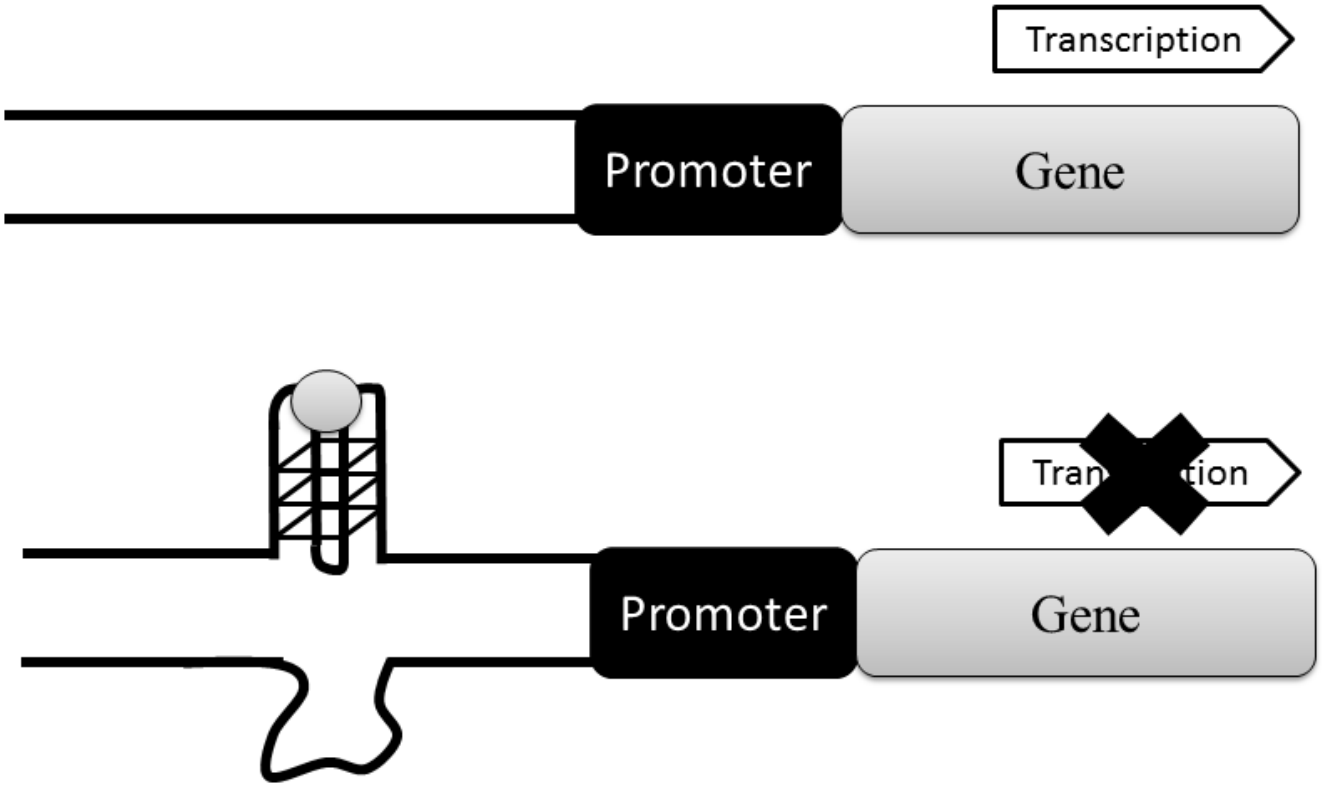
(Δ pH, Δ ion strengh, ion type, MC, hydration)

… even helix geometry is controlled by environment



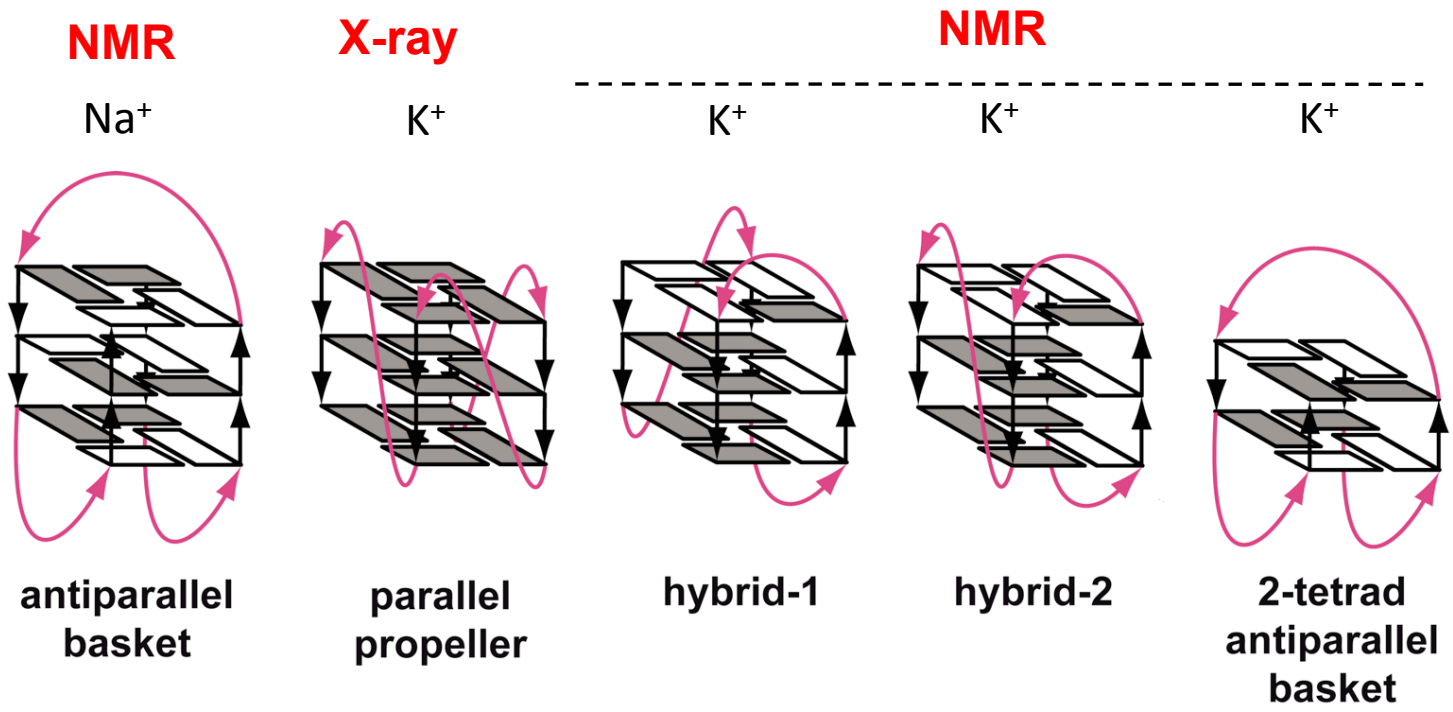
Vargason et al. PNAS 2001

Polymorphism as a source of “targets”



Polymorphism as a source of "problems" in the process of drug development

Architecture of telomeric in G-rich single stranded 3'-overhang - d(TTAGGG)n



Wang et al. ***Structure*** *(1993)*

Ambrus et al. ***Nucleic Acids Res.*** *(2006)*

Parkinson et al. ***Nature*** *(2002)*

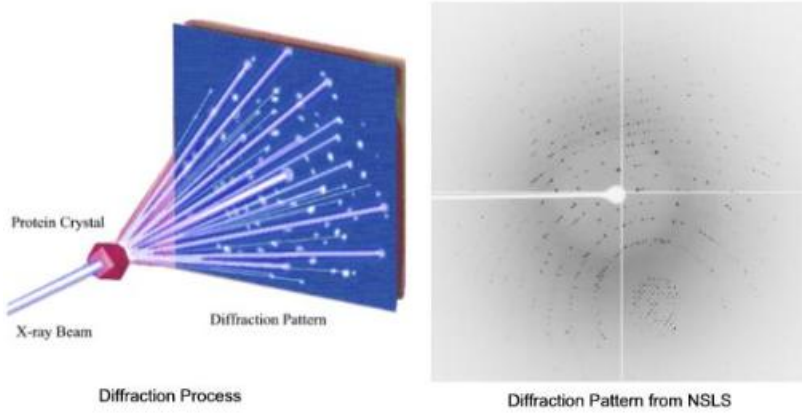
Dai et al. ***Nucleic Acids Res.*** *(2007)*

Lim et al. ***J Am Chem Soc.*** *(2009)*

Structural Biology of NA – an issue

How to recognize physiologically relevant structure

X-ray diffraction



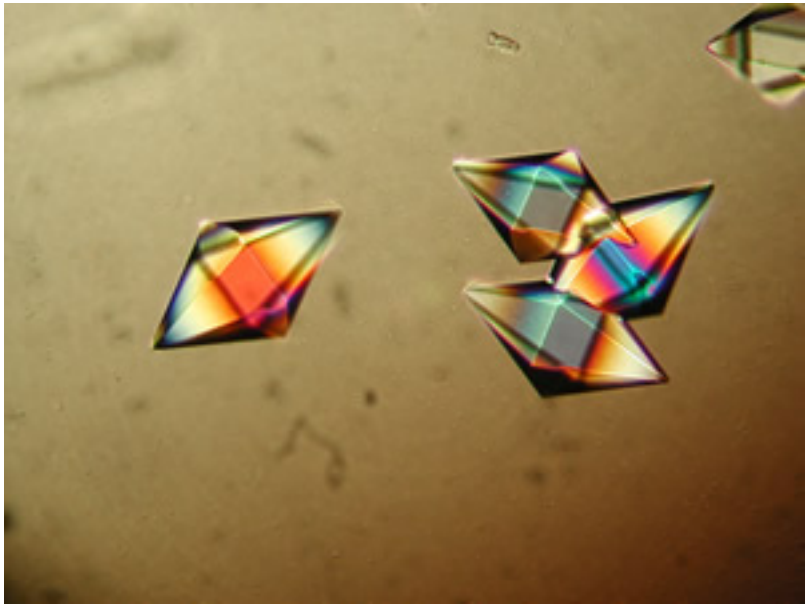
Diffraction Process

Diffraction Pattern from NSLS

… thus far, it is not possible to detect diffraction from single molecule☹

X-ray diffraction relies on

monocrystal production



X-ray diffraction &

monocrystal production

Crystal Screen™

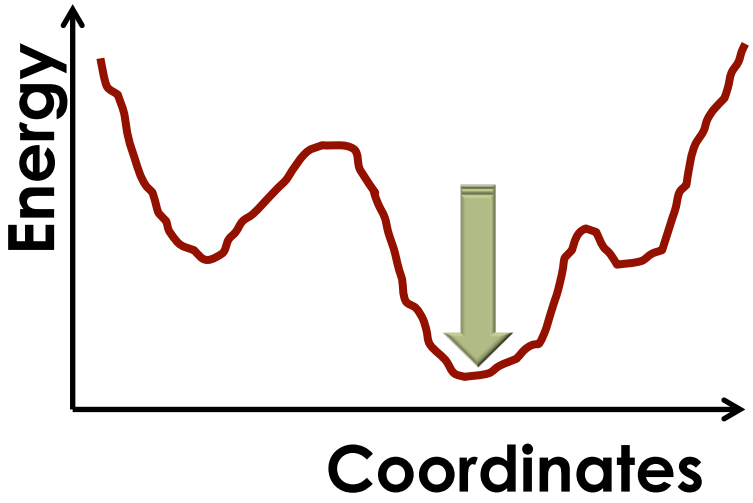
HR2-110 Reagent Formulation

Tube #	Salt	Buffer ◊	Precipitant
1.	0.02 M Calcium chloride dihydrate	0.1 M Sodium acetate trihydrate pH 4.6	30% v/v (+/-)-2-Methyl-2,4-pentanediol
2.	None	None	0.4 M Potassium sodium tartrate tetrahydrate
3.	None	None	0.4 M Ammonium phosphate monobasic
4.	None	0.1 M TRIS hydrochloride pH 8.5	2.0 M Ammonium sulfate
5.	0.2 M Sodium citrate tribasic dihydrate	0.1 M HEPES sodium pH 7.5	30% v/v (+/-)-2-Methyl-2,4-pentanediol
6.	0.2 M Magnesium chloride hexahydrate	0.1 M TRIS hydrochloride pH 8.5	30% w/v Polyethylene glycol 4,000
7.	None	0.1 M Sodium cacodylate trihydrate pH 6.5	1.4 M Sodium acetate trihydrate
8.	0.2 M Sodium citrate tribasic dihydrate	0.1 M Sodium cacodylate trihydrate pH 6.5	30% v/v 2-Propanol
9.	0.2 M Ammonium acetate	0.1 M Sodium citrate tribasic dihydrate pH 5.6	30% w/v Polyethylene glycol 4,000
10.	0.2 M Ammonium acetate	0.1 M Sodium acetate trihydrate pH 4.6	30% w/v Polyethylene glycol 4,000
11.	None	0.1 M Sodium citrate tribasic dihydrate pH 5.6	1.0 M Ammonium phosphate monobasic
12.	0.2 M Magnesium chloride hexahydrate	0.1 M HEPES sodium pH 7.5	30% v/v 2-Propanol
13.	0.2 M Sodium citrate tribasic dihydrate	0.1 M TRIS hydrochloride pH 8.5	30% v/v Polyethylene glycol 400

X-ray diffraction

… underlying assumptions

- **Lowest energy structure is biologically active**



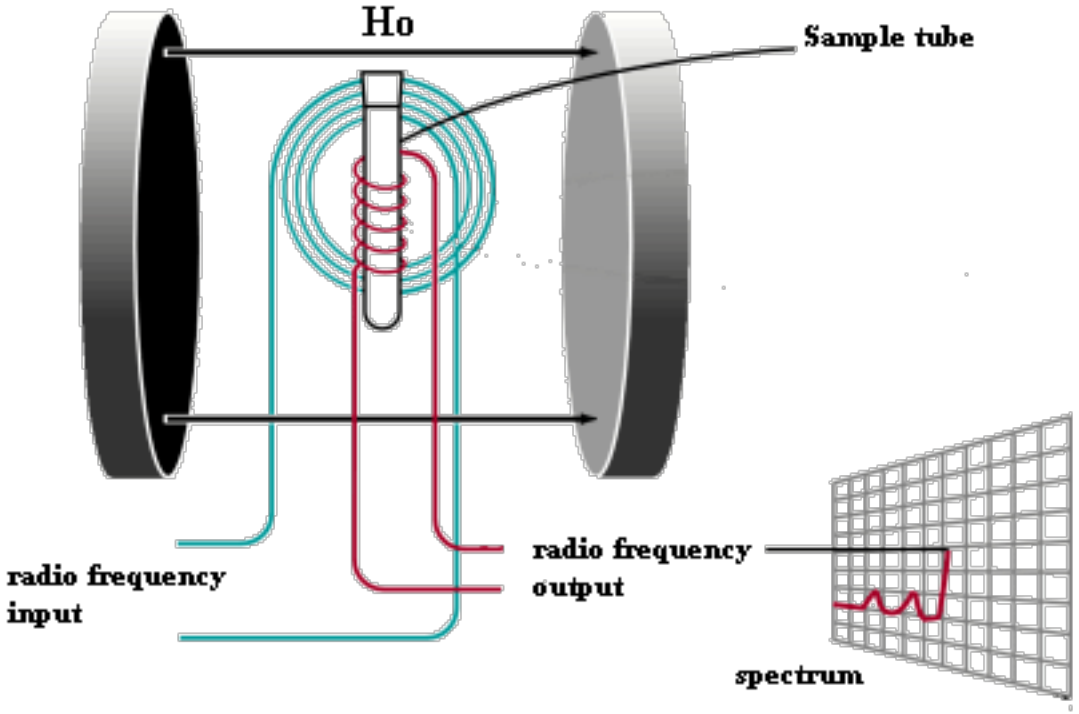
- **structure is independent of environmental conditions**

additives, hydration levels, temperature, MC, viscosity, concentration, ion type, ion strength, ……

PDB statistics

Exp. Method	Proteins	Nucleic Acids	Protein-NA complexes	Other
X-ray	75 215	1 464	3 888	2
NMR	8 737	1 030	192	7
El. Microsc.	428	45	128	0
Hybrid	46	3	2	1
Other	148	4	6	13
Total	84 574	2 546	4 216	23

Nuclear Magnetic Resonance

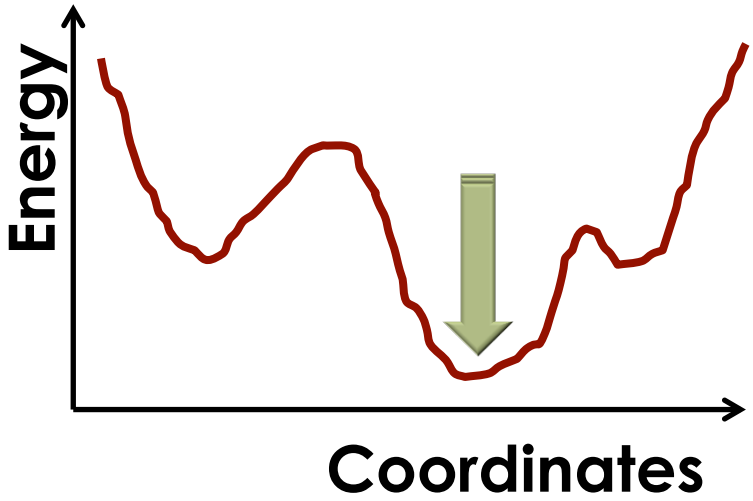


Sample: water based solutions, [biomolecule] ~ 50 – 3 mM, Te ~ 0 – 45 °C

NMR spectroscopy

… underlying assumptions

- **Lowest energy structure is biologically active**
  (in principle NMR also allows determination of high energy states)

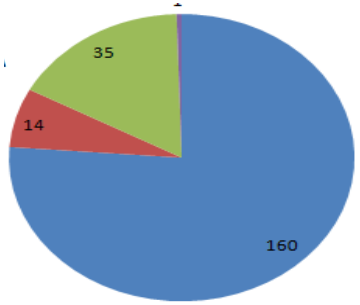


NMR spectroscopy

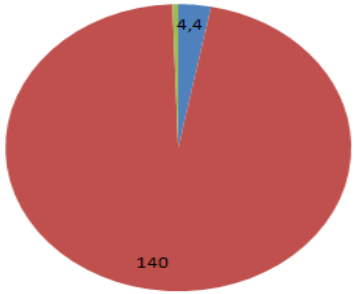
… can be physiological, but …

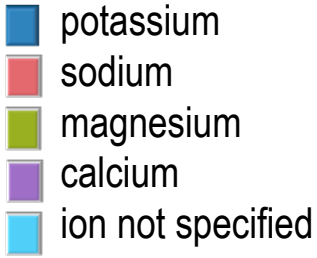
Ionic composition of:

Intracellular space

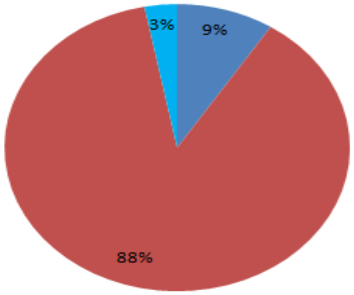


Extracellular space

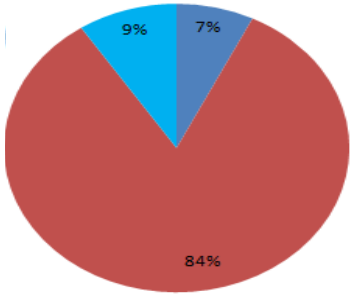




Ionic composition of buffers used for NMR studies of:



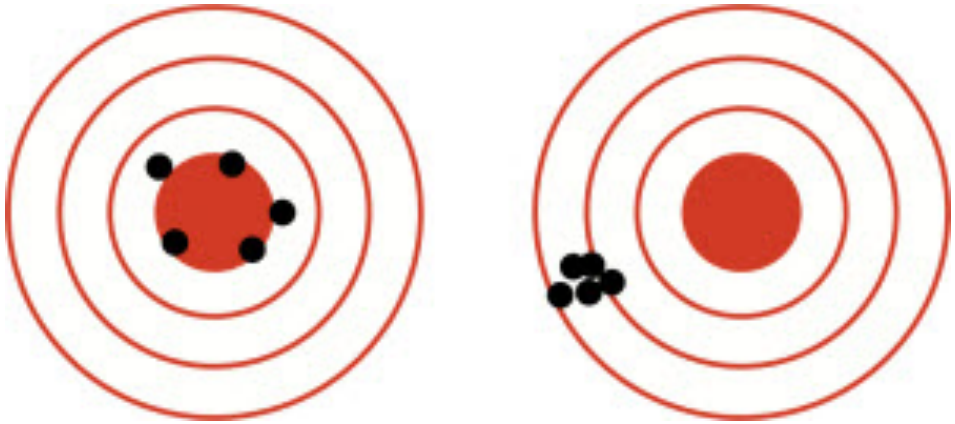
DNA



RNA

Structural Biology – an issue

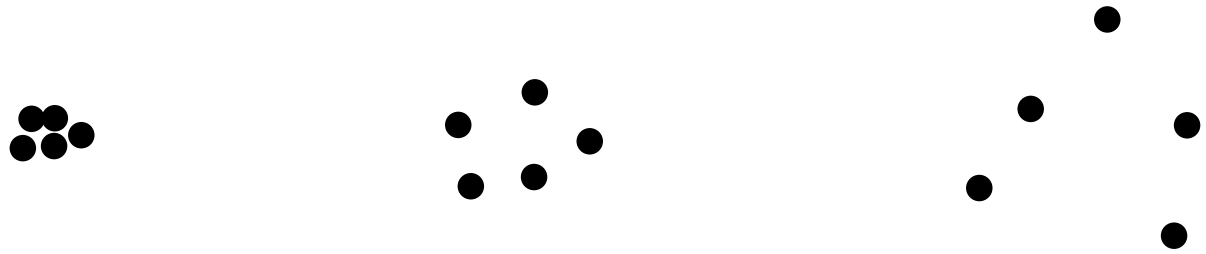
Precision vs. accuracy



Structural Biology – an issue

conventional NMR as well as X-ray

.. are only able to assess structure precision



X-ray - Resolution

NMR spectroscopy - RMSD

.. NOT its accuracy

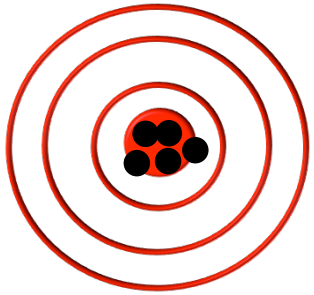
Dark secret of structural biology

X-ray & NMR “shoot” without knowing where the target is

… assessment of structural accuracy presumes knowledge of reference structure

Cellular Structural Biology

… on target shooting

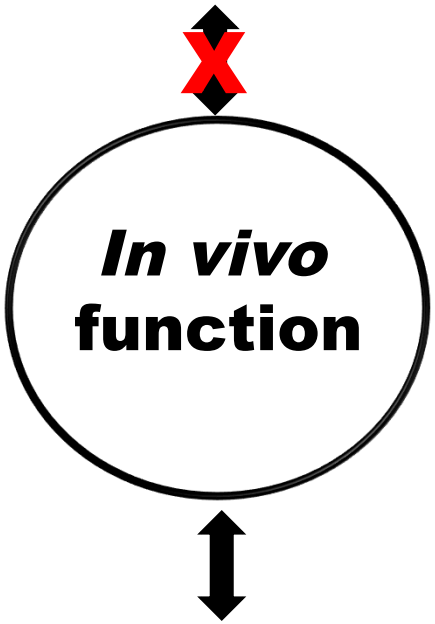


Cellular Structural Biology – a concept

How to find a "target"

***In vitro* structure & dynamics**

buffered solutions or crystalline state



***In vivo* structure & dynamics**

Complex environment of living cells

Cellular Structural Biology - proteins

– a history

Proteins

2000

- in-cell NMR of proteins **overexpressed** in bacterial cells

2006

- In-cell NMR of proteins **delivered** into X. laevis oocytes

2009

- In-cell NMR of **delivered** proteins in mammalian cells; 1st high resolution structure of protein inside living cells

2011 …

- In-cell NMR of proteins **overexpressed** in yeast, insect cells, mammalian cells

2012 …

- In-cell EPR of proteins **delivered** in bacteria, X. oocytes

Cellular Structural Biology

– a history

Nucleic Acids

2009
- in-cell NMR: DNA/RNA injected in X. laevis oocytes

2010
- In-cell EPR: DNA injected in X. laevis oocytes

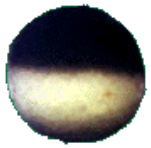
2012
- In-cell spFRET: DNA in bacterial and mammalian cells

…. **all delivered**

In-cell NMR of nucleic acids

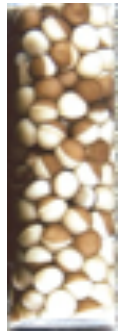
NA delivery via mechanical injection

Xenopus laevis

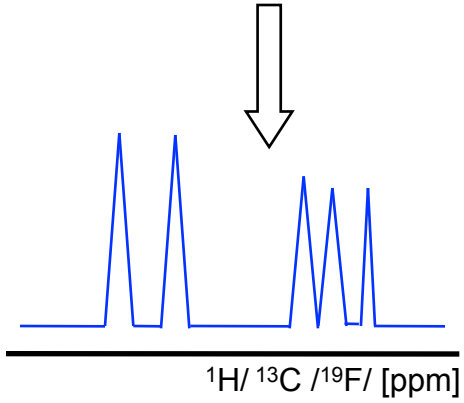


Stage IV oocyte

Injection – 50 nl/oocytes, [NA] 100-250uM

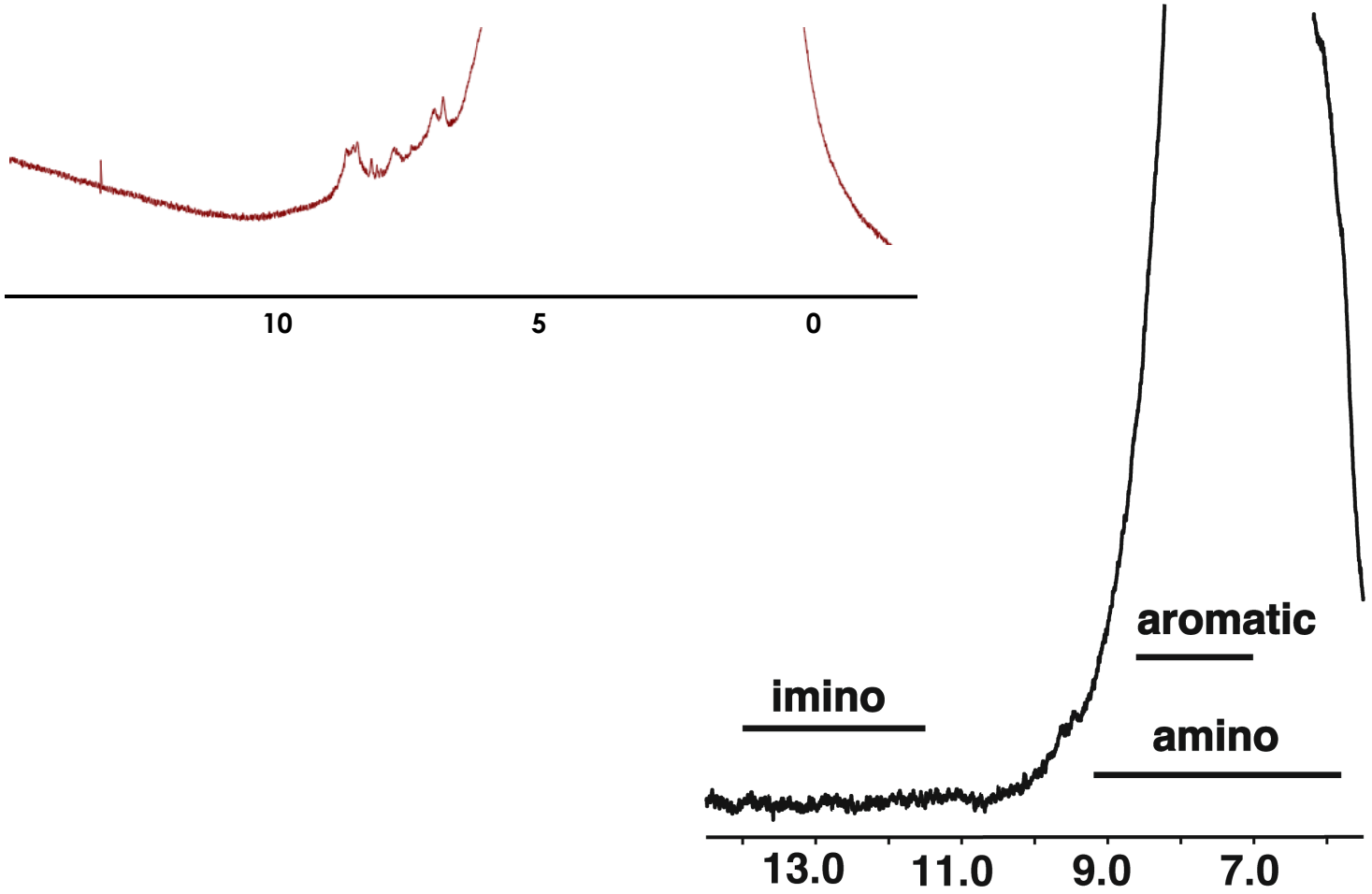


~ 200 oocytes/sample

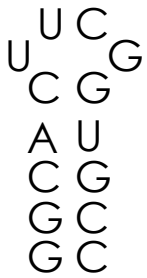


Hänsel et al. ***J Am Chem Soc.*** 2009

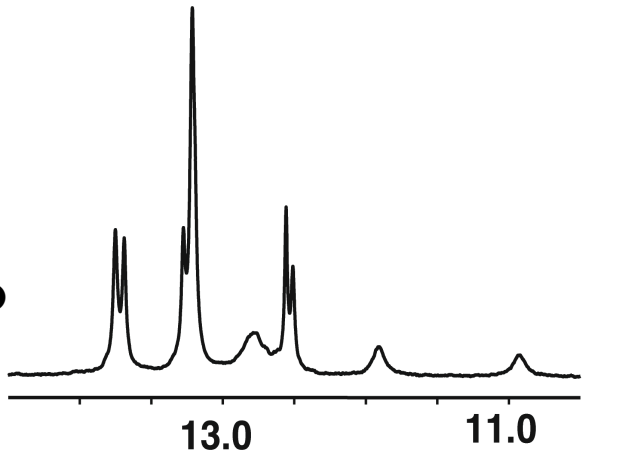
Signals from NA vs. (friendly) cellular background



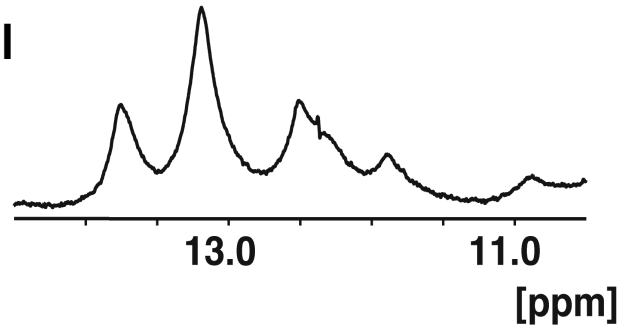
Topology information from imino pattern

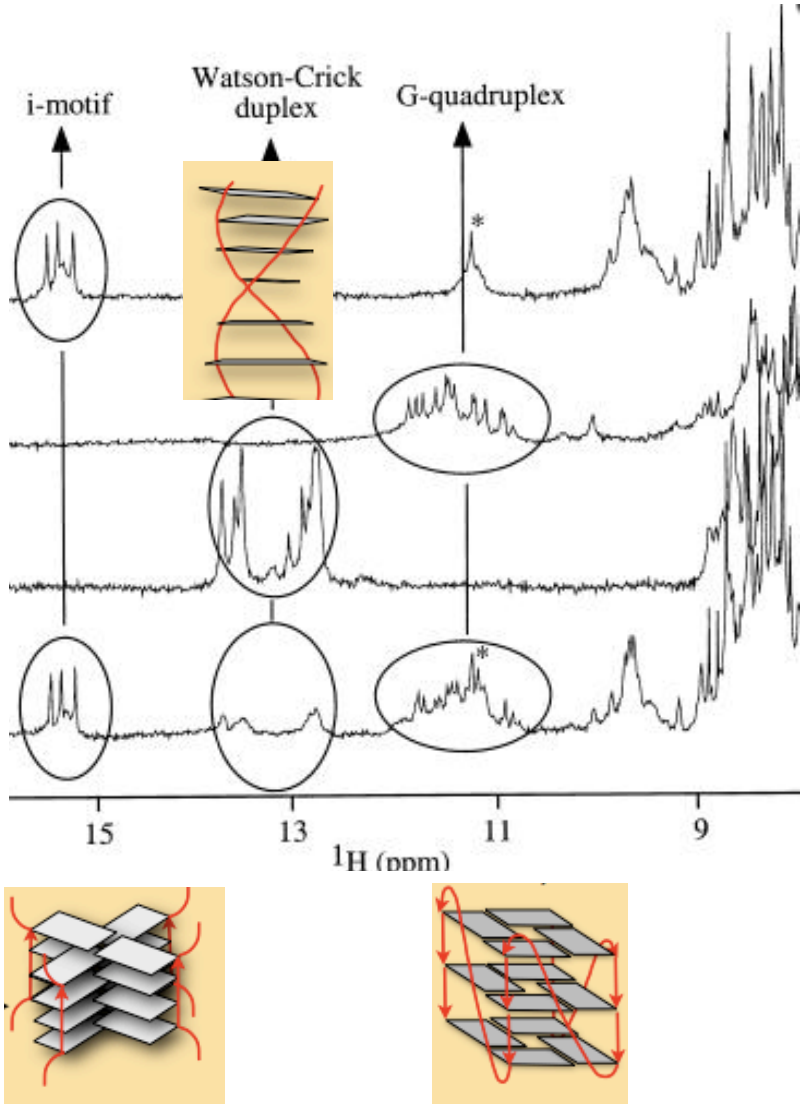


In vitro

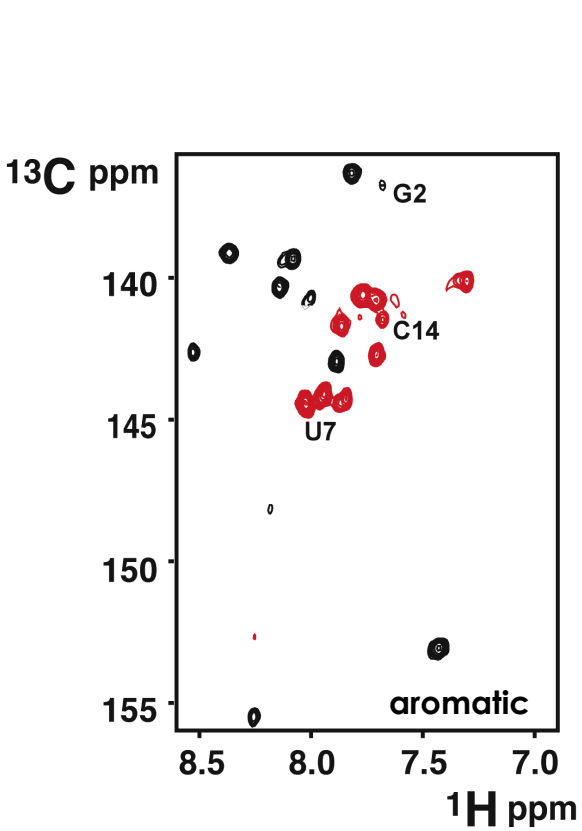


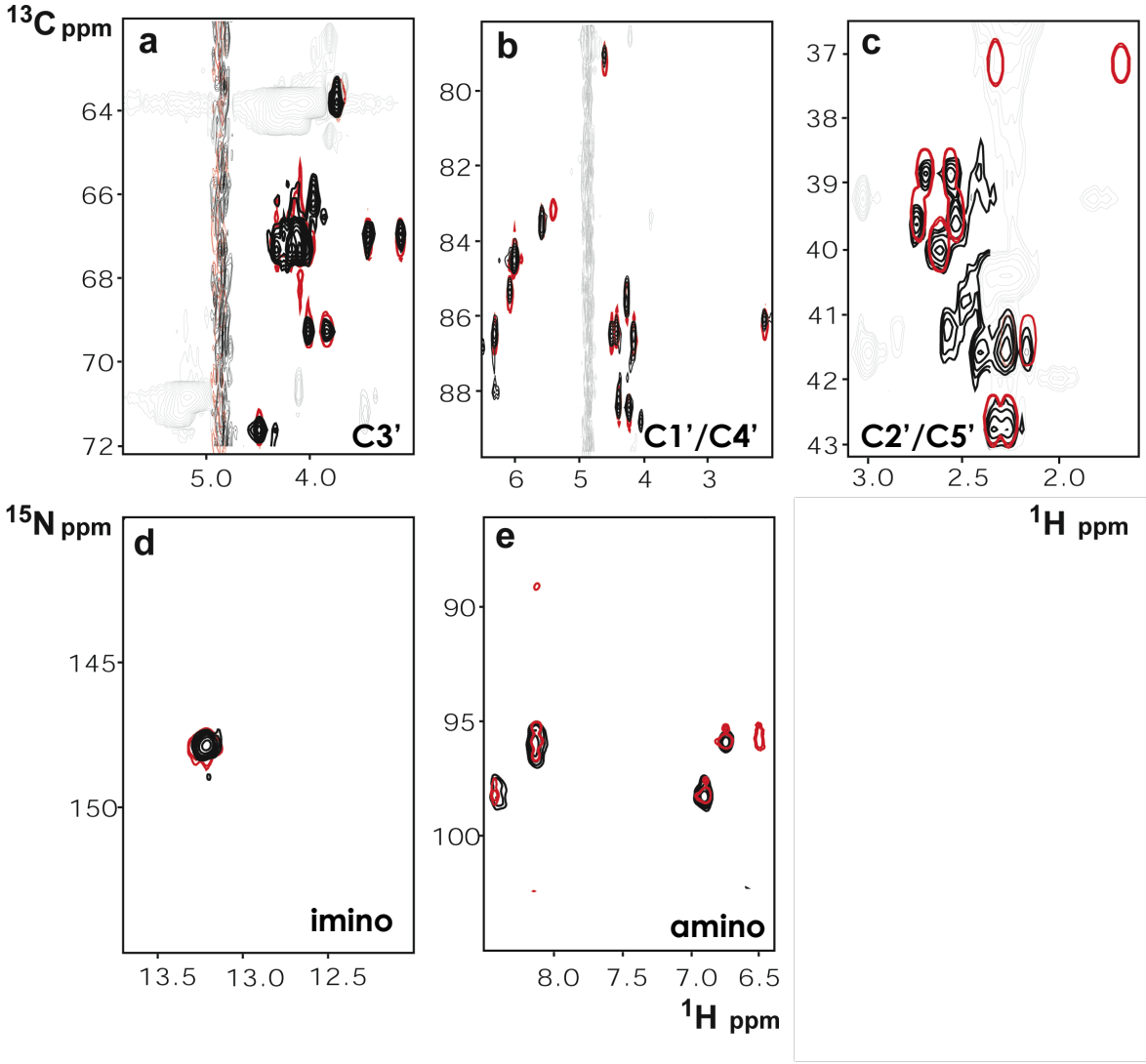
In cell



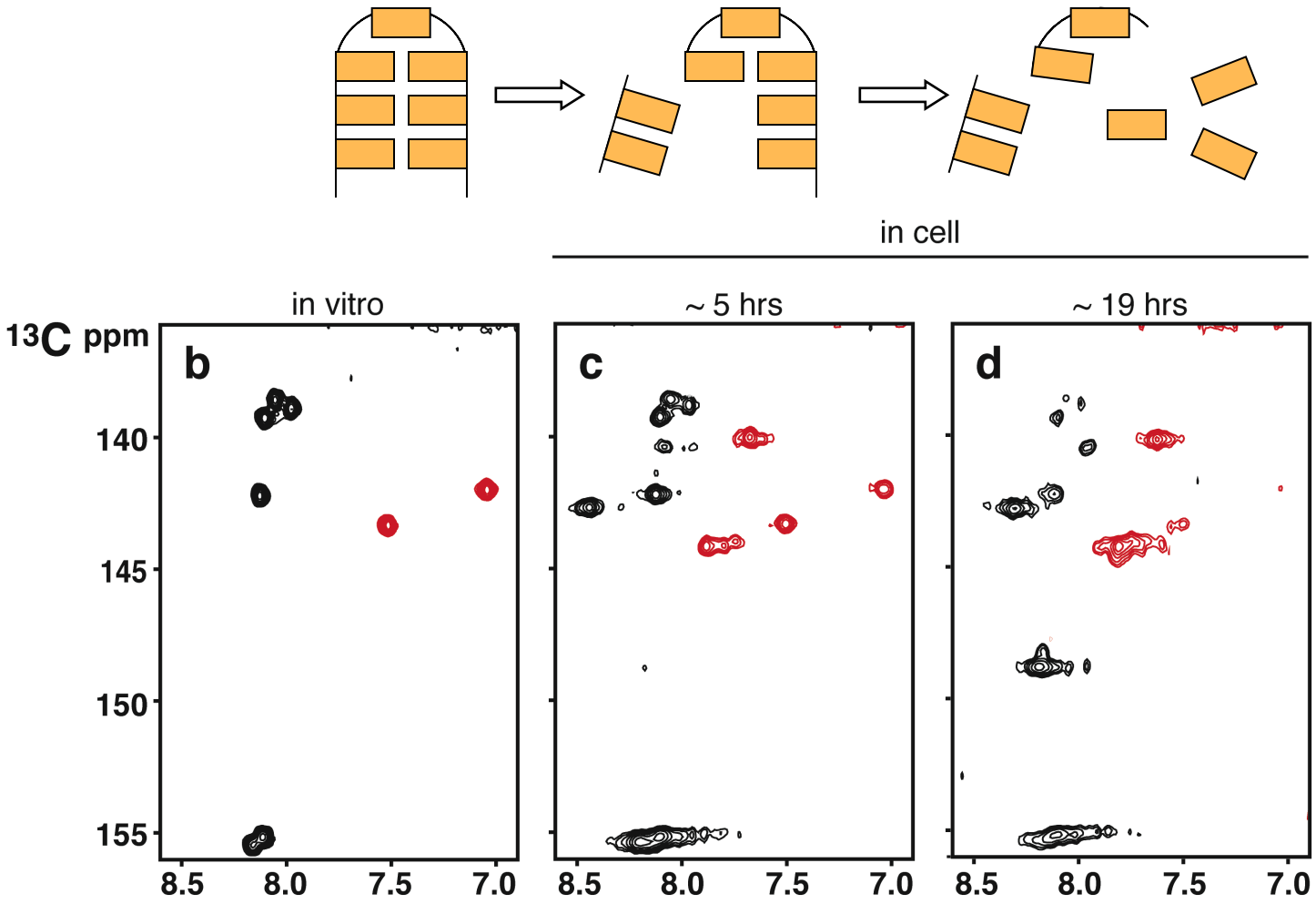


To see the rest - isotopically labeled samples

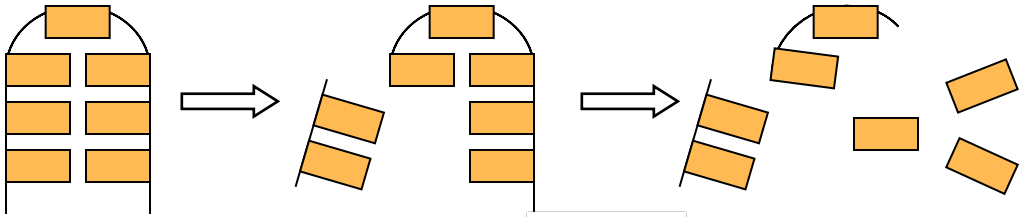


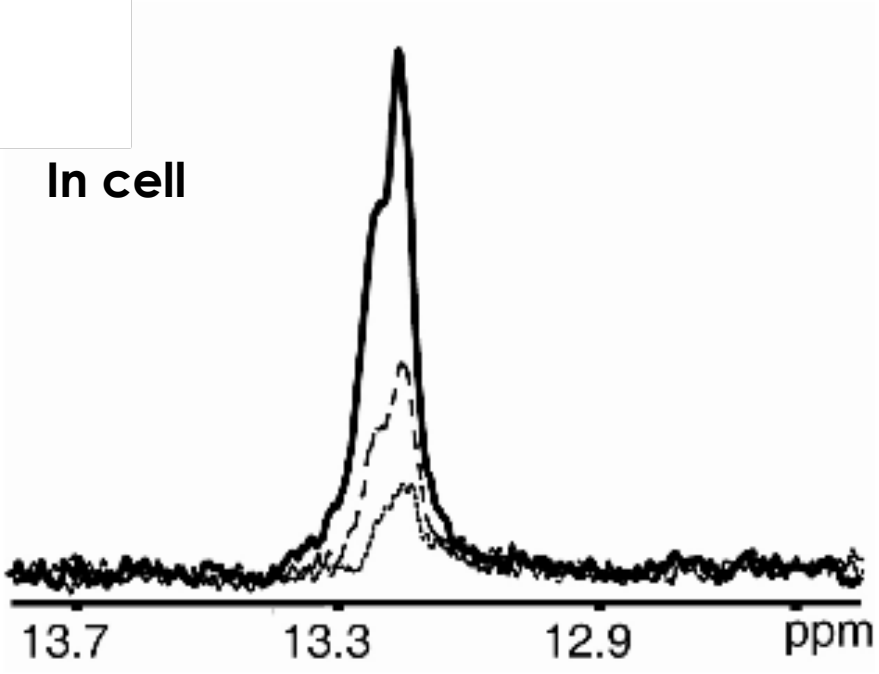


In-cell NMR: NA degradation (un)expected problem



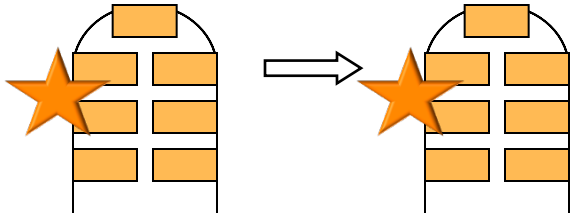
In-cell NMR: NA degradation (un)expected problem

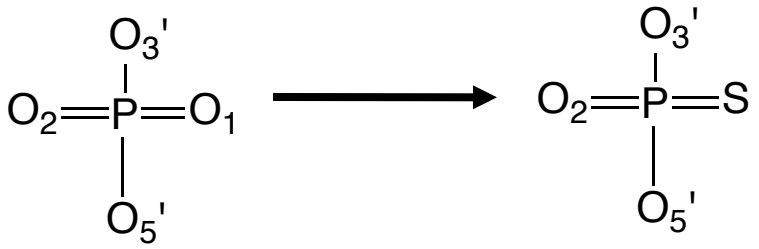


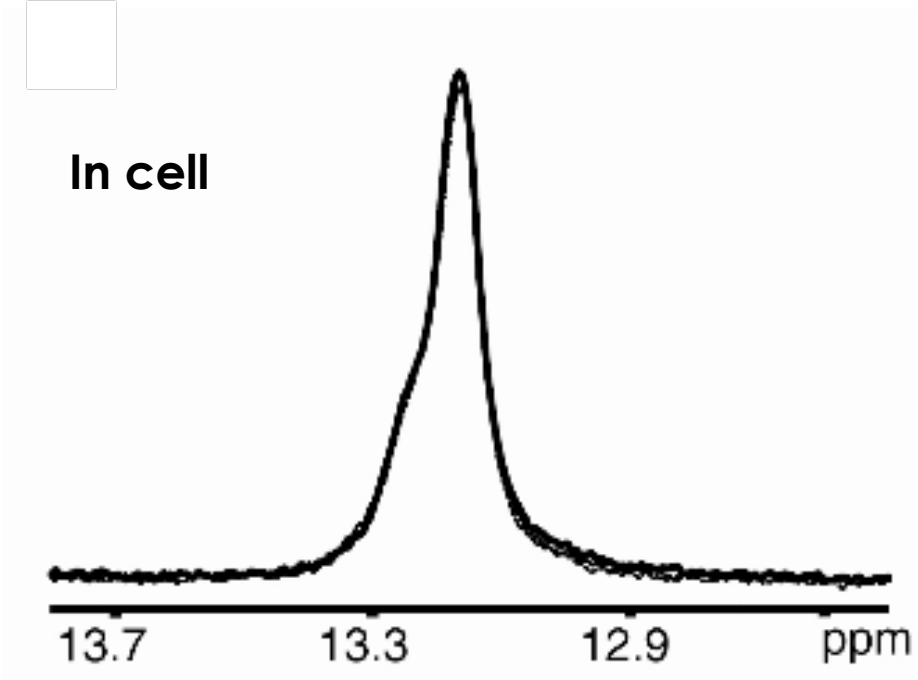


30 min 180 min 360 min

Chemical stabilization prevents NA degradation

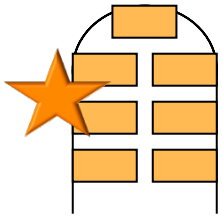


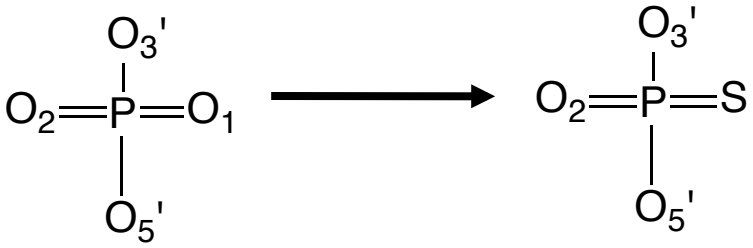




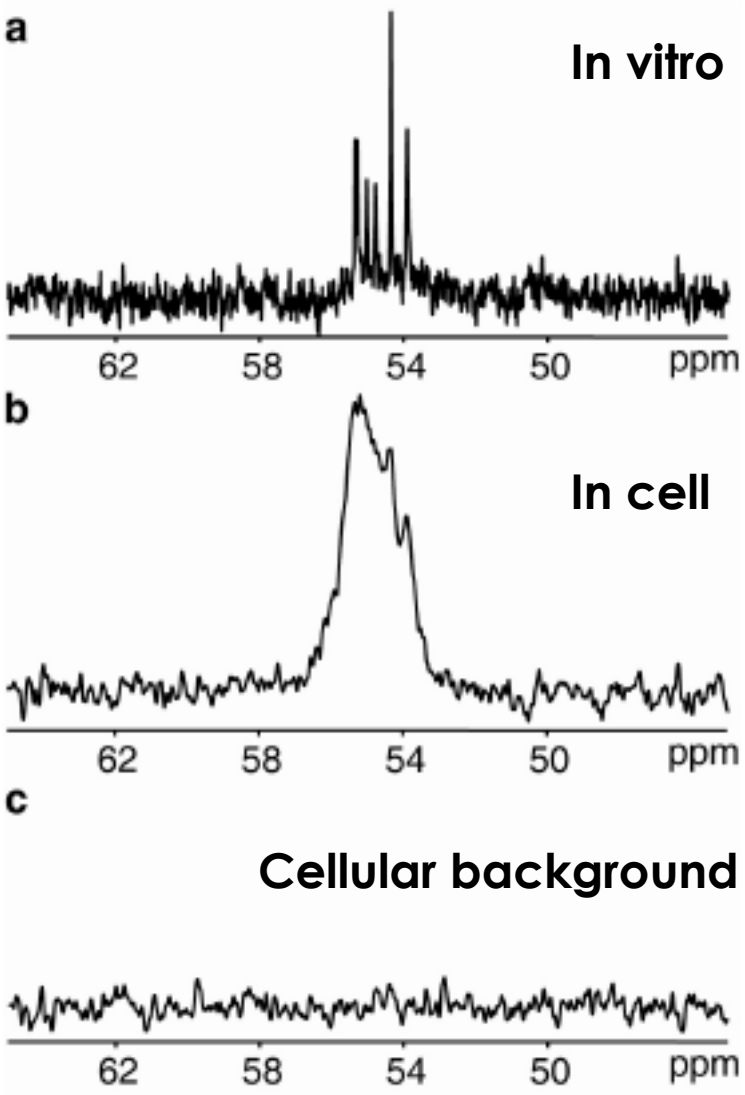
30 min 180 min 360 min

Phosphotioester moiety allows monitoring the NA backbone

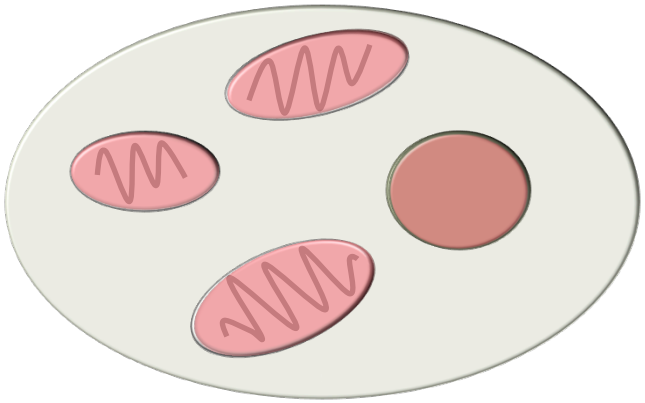




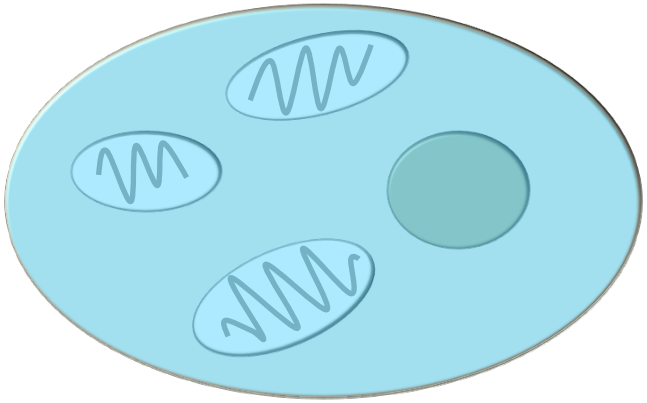
In-cell 1D ^{31}P NMR spectra



Problem of intracellular localization

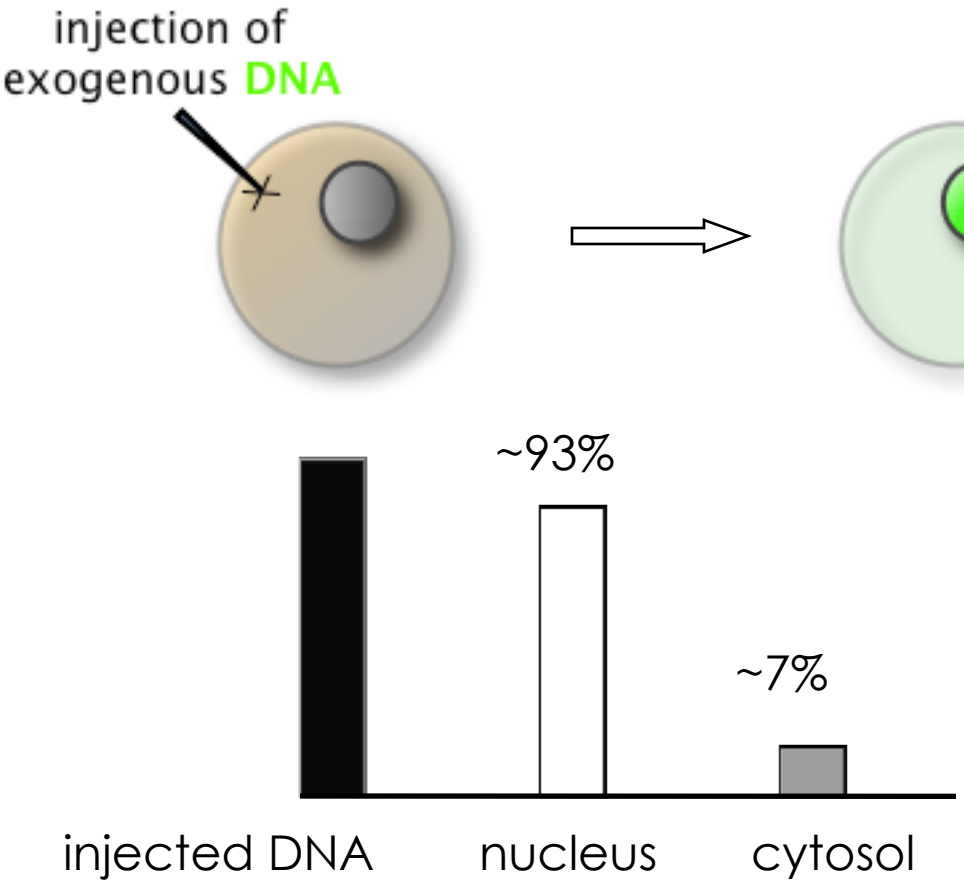


DNA

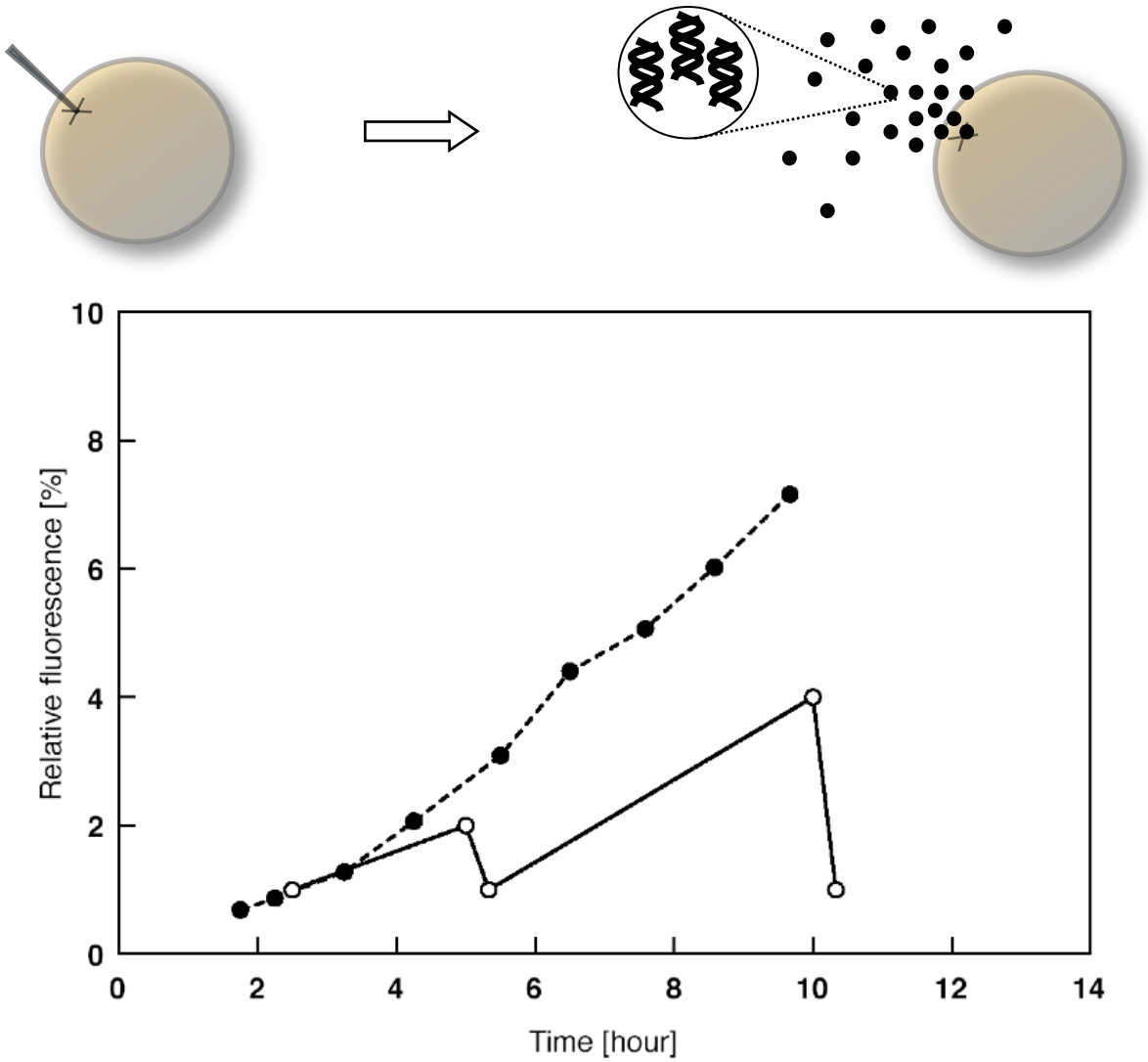


RNA

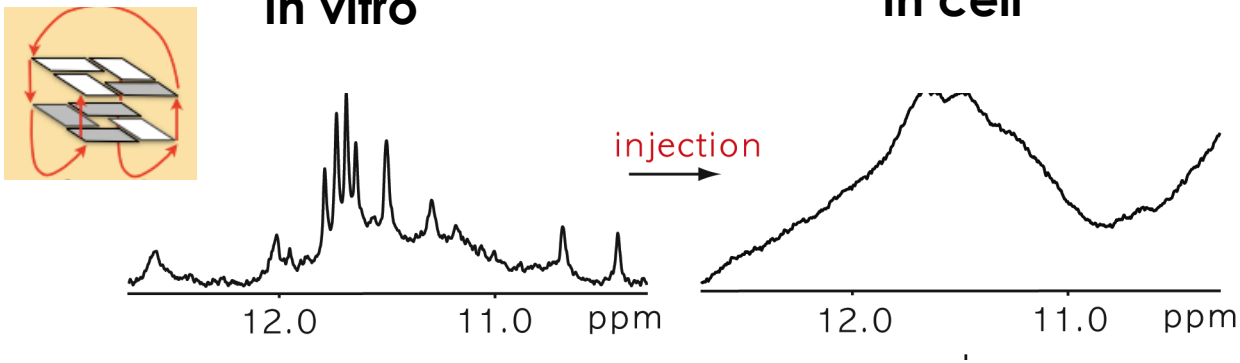
Introduced DNA localizes in nucleus



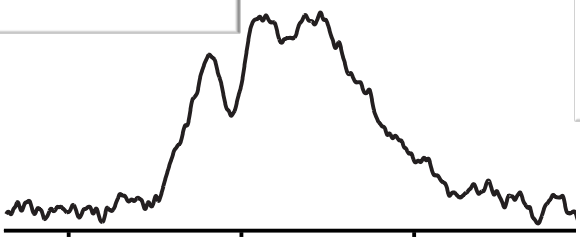
In-cell NMR: NA leakage from incisions



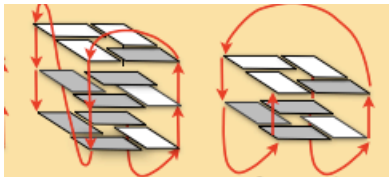
Resolution limits the analysis of the polymorphs: Cellular lysates

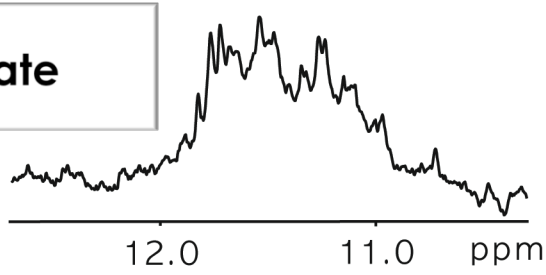


Crude cellular lysate



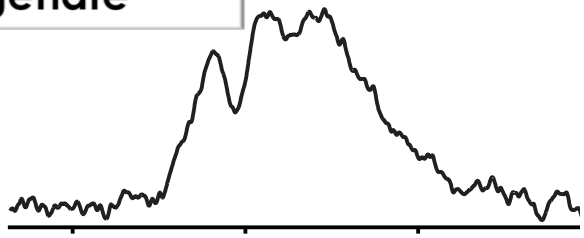
Cleared lysate



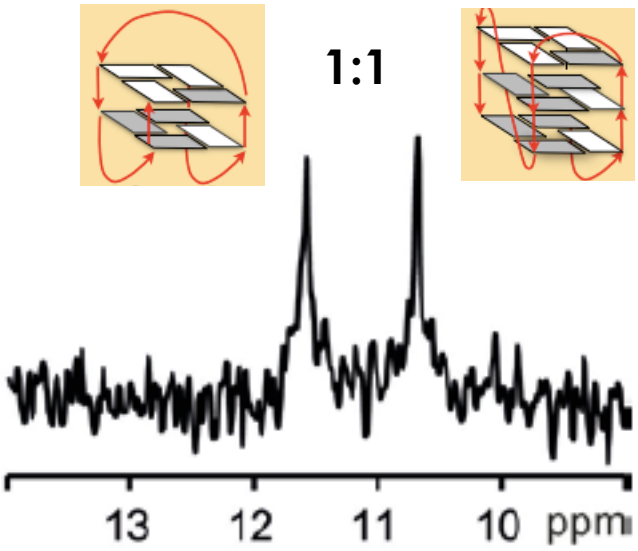


Resolution limits the analysis of the polymorphs: site-specific labeles

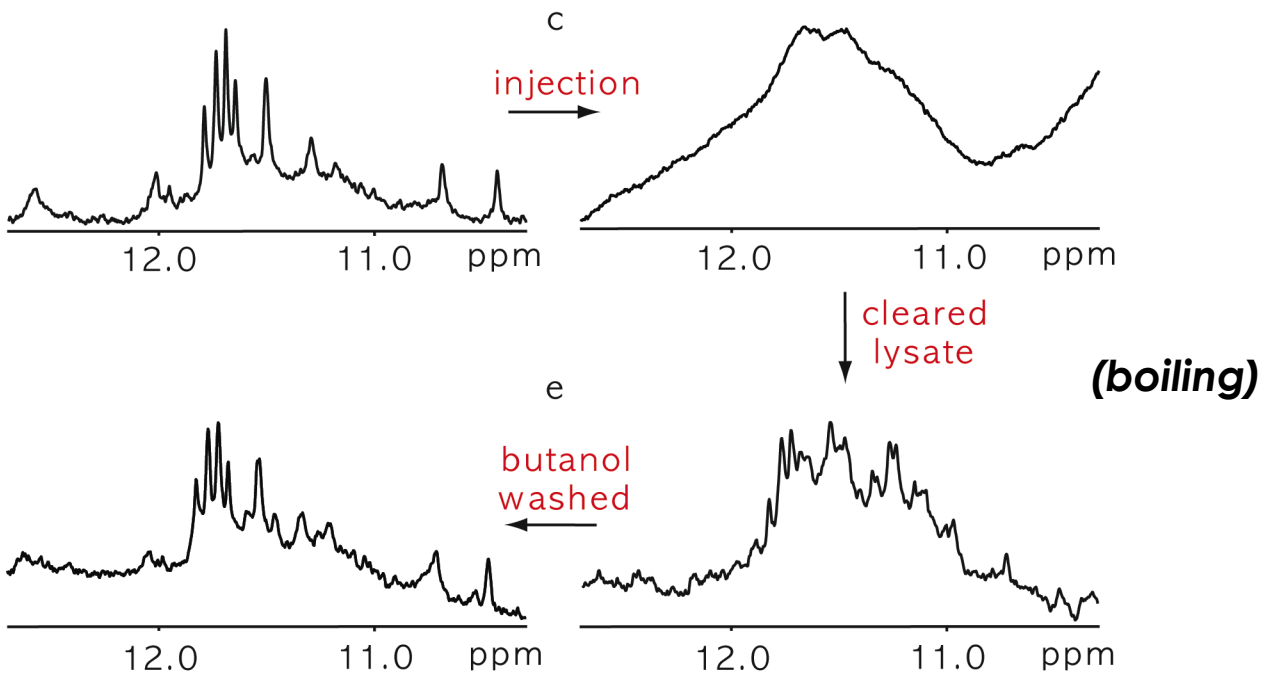
Crude cellular homogenate



GGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTAGGGTTA



a good news: DNA/RNA (if there is any) can be recovered from cells



Summary: in-cell NMR of NA

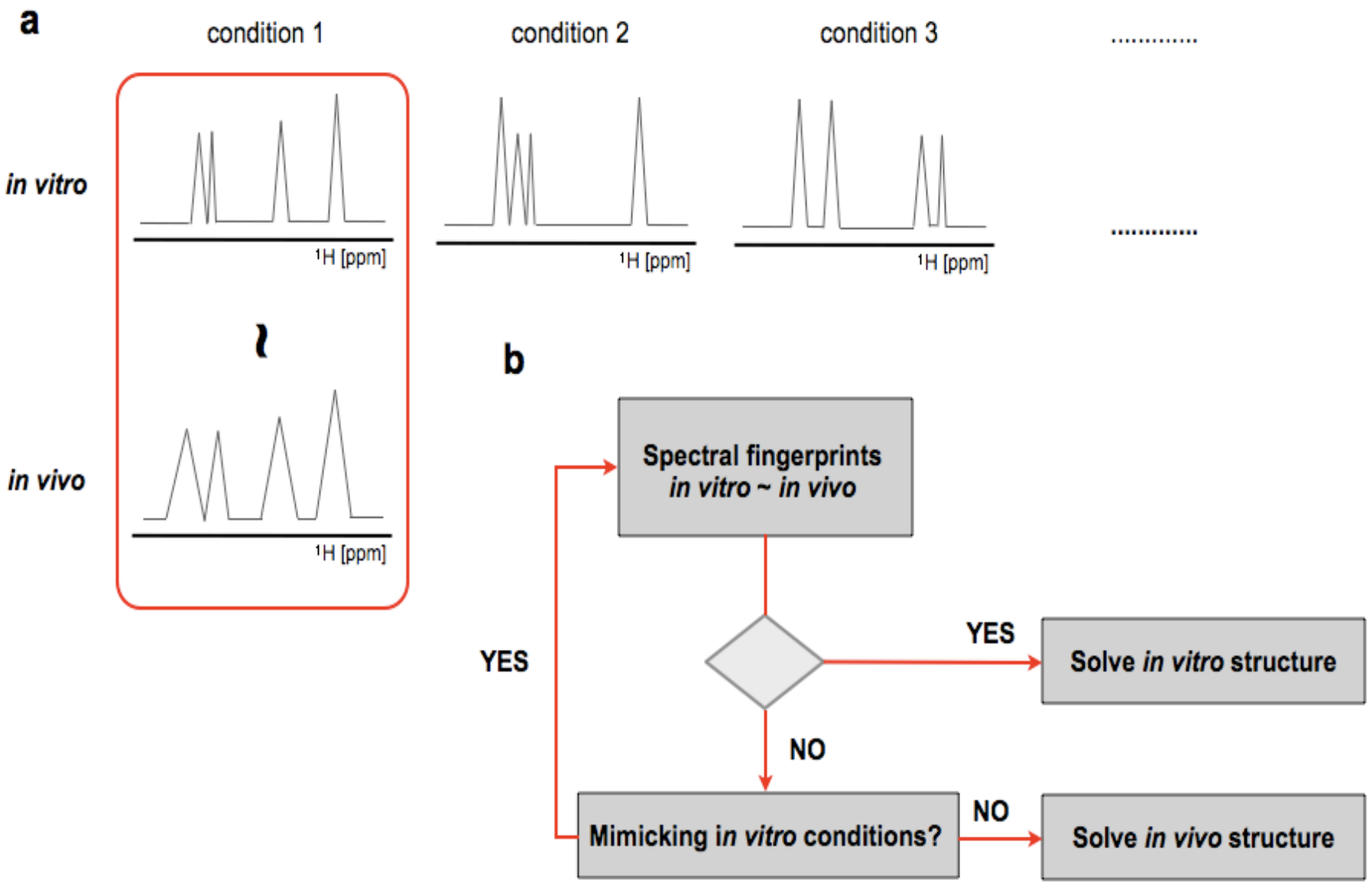
… NA can be studied inside eukaryotic cells at atomic resolution

- <25 without isotopic labeling (imino H/secondary structure)
- with isotopic labeling up to 70 nt
- degradation can be diminished via chemical modification
- experimental time-window < 3 (leakage, degradation)

Application potential:

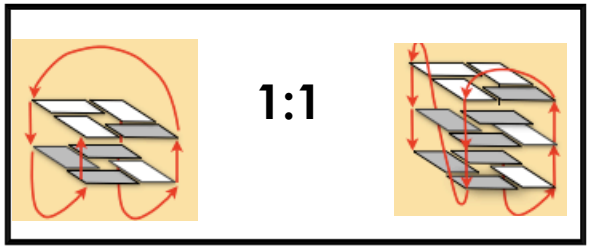
- *de novo* structure determination – limited (price-wise)
- fold validation - YES
- NA sensitivity to environmental factors – YES
- DNA drug interactions – YES (Selgado & Mergny)

Interpretation of in-cell NMR data: spectral fingerprinting

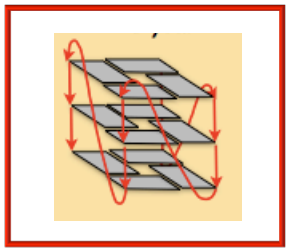


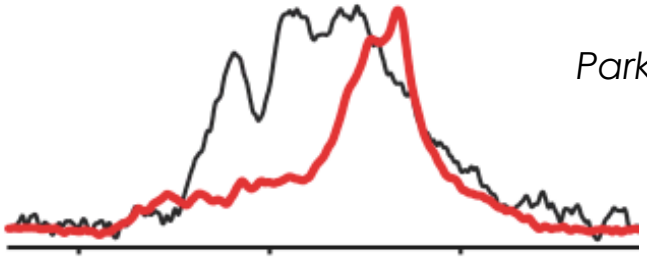
Spectral fingerprinting: example

Crude cellular homogenate



X-ray

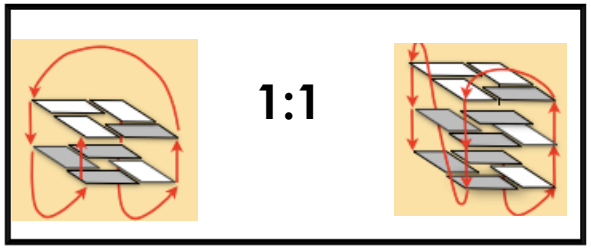




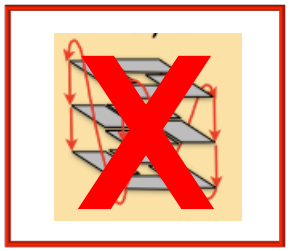
Parkinson et al. ***Nature*** *(2002)*

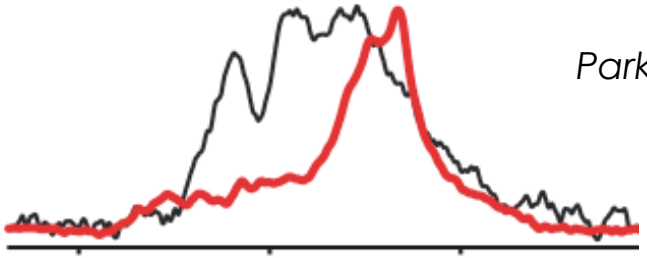
Spectral fingerprinting: example

Crude cellular homogenate



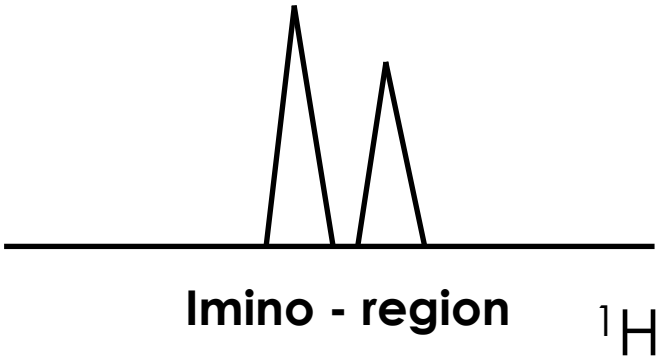
X-ray



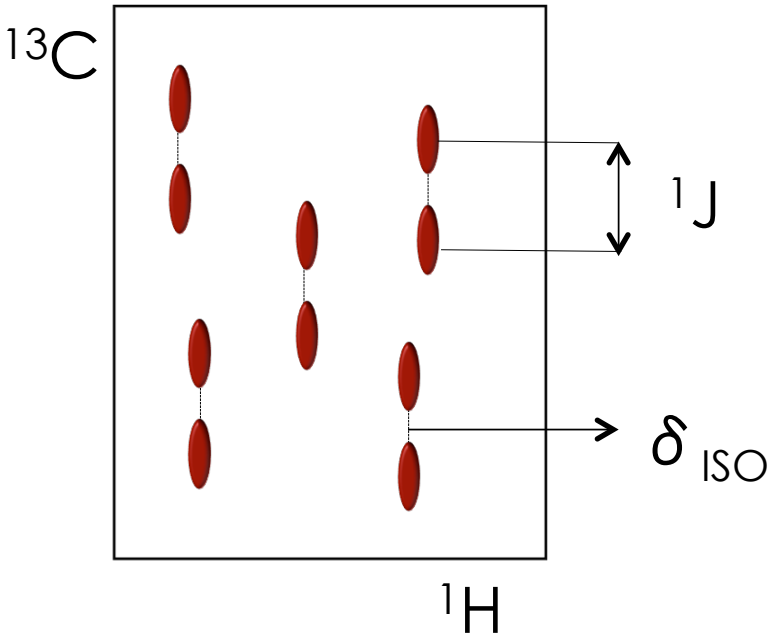


Parkinson et al. ***Nature*** *(2002)*

Benchmarking of in-cell NMR spectra to NA motifs



Base-pairing pattern
(WC/Hoogsteen/i-motif)

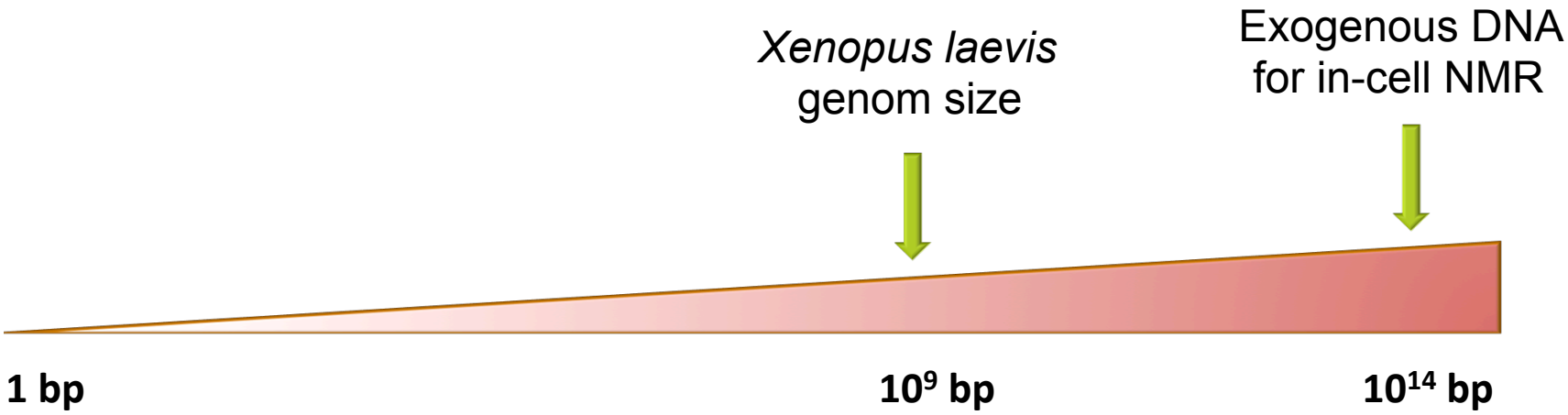


Sugar pucker (C2-endo/C3-endo)

Glycosidic torsion angle (syn/anti)

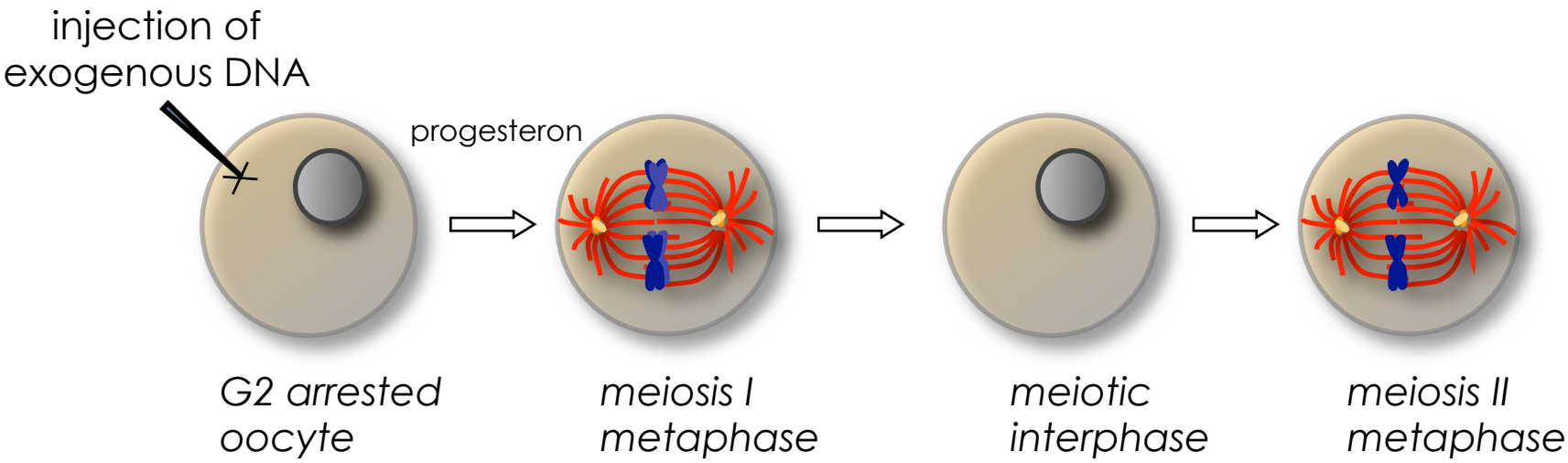
Stacking (A-like/B-like)

Does “being in cell” means “being native”?



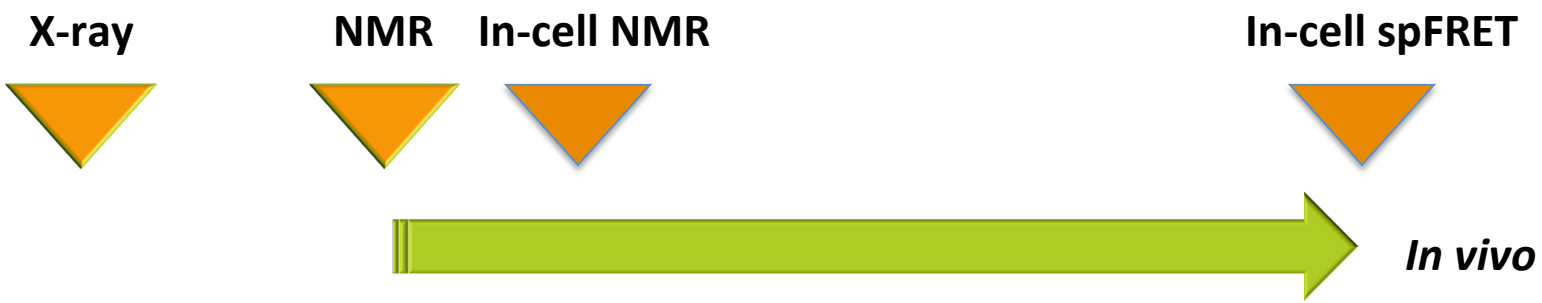
Unnaturally high concentration of NA are introduced

Injected cells propagate through meiosis



Cells accommodate/tolerate introduced NA

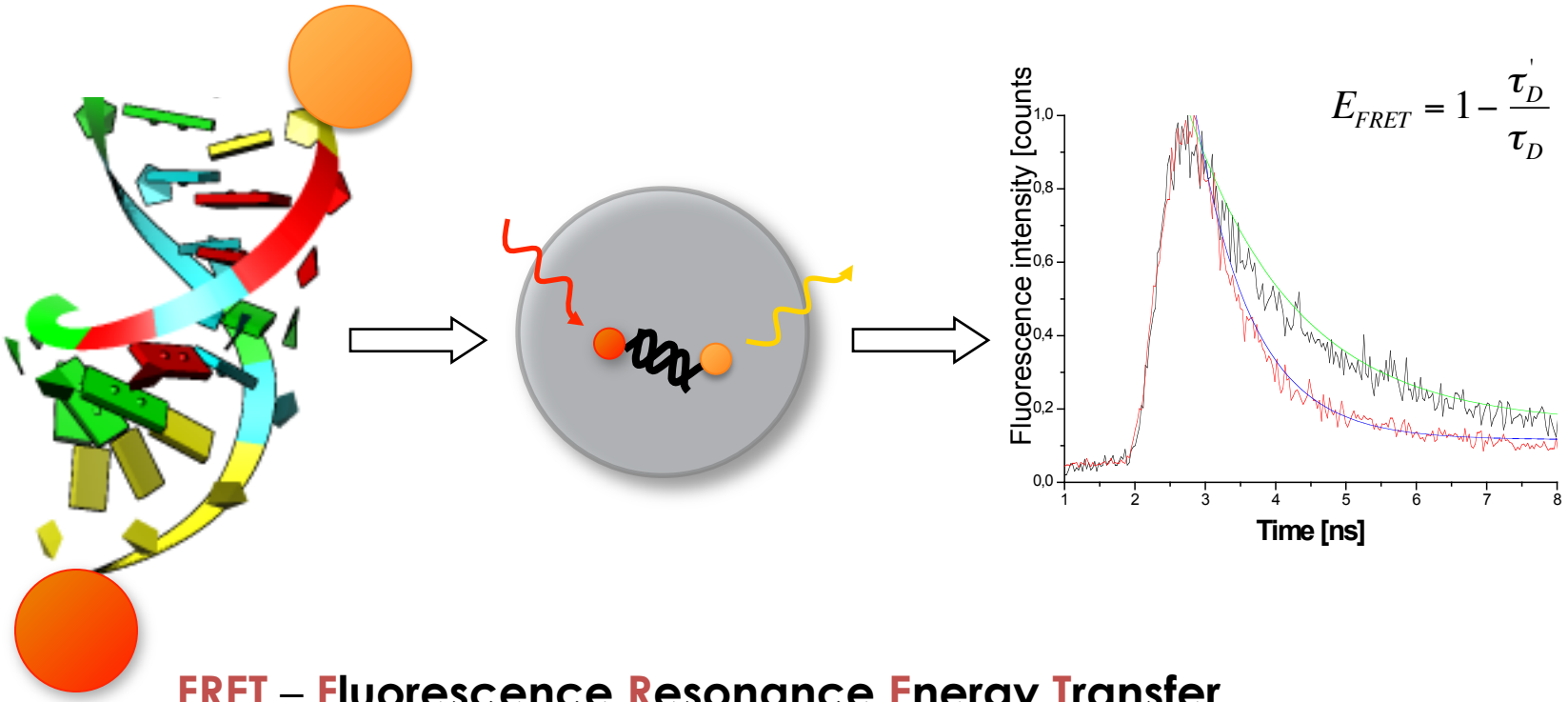
Towards structural biology under native conditions…



In-cell single particle FRET

DNA/RNA

cell

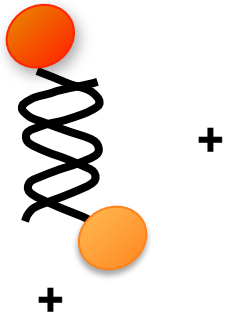


FRET – Fluorescence Resonance Energy Transfer

Fessl et al ***Nucl Acids Res***. 2012

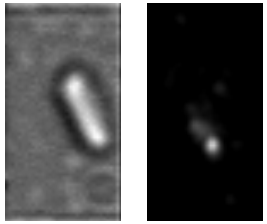
In-cell single particle FRET

E. coli

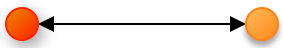


Growth medium

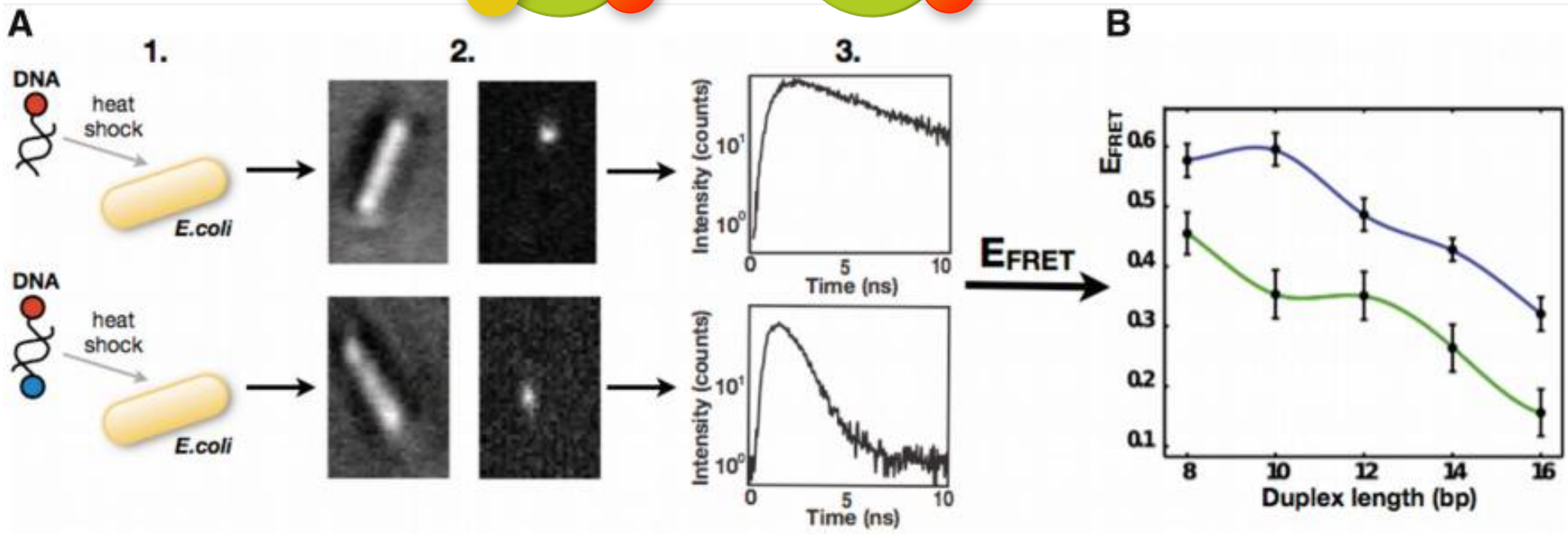
Heat shock



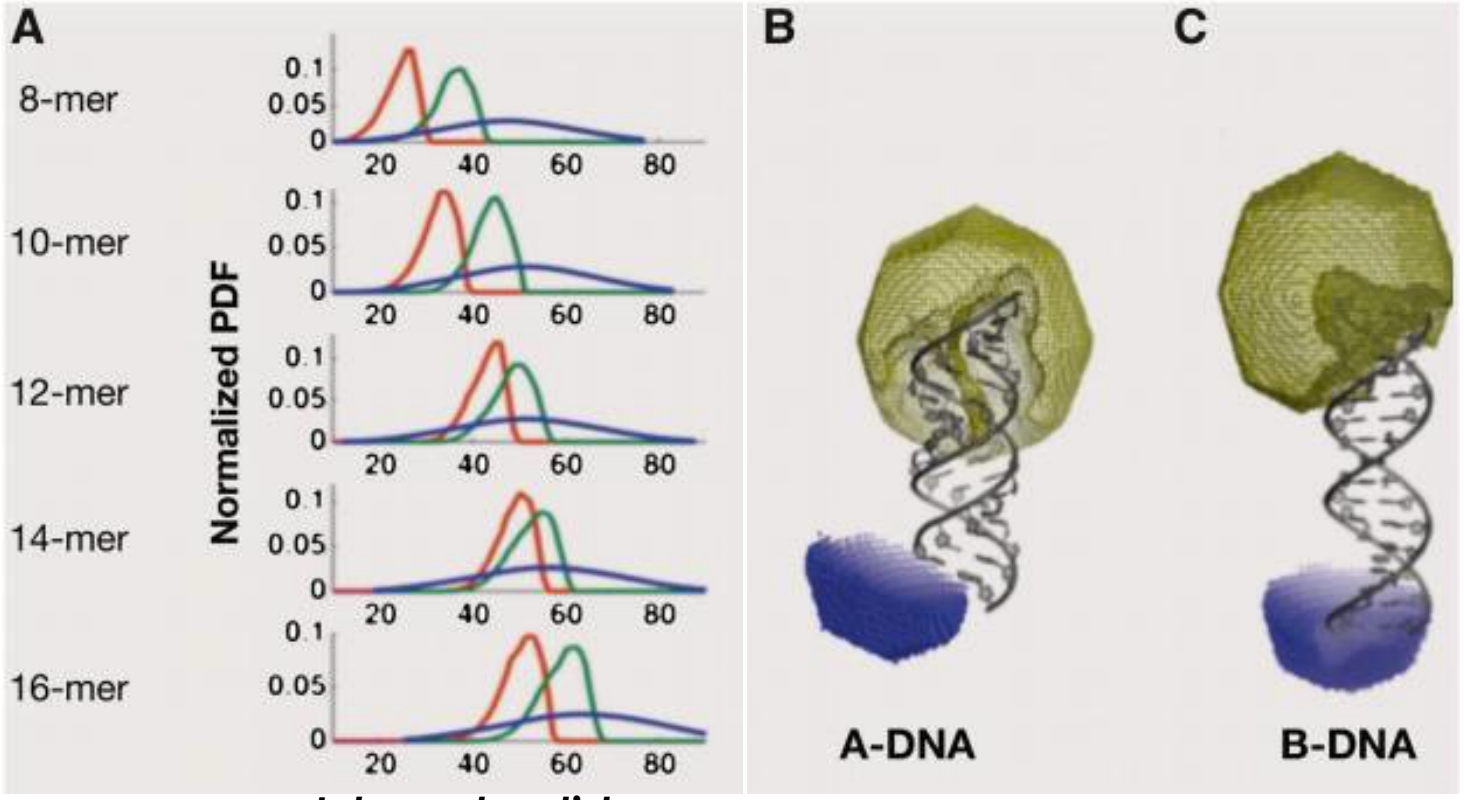
D/A distance [nm]



Interepretation based on rigid arrangement of tags might be biased



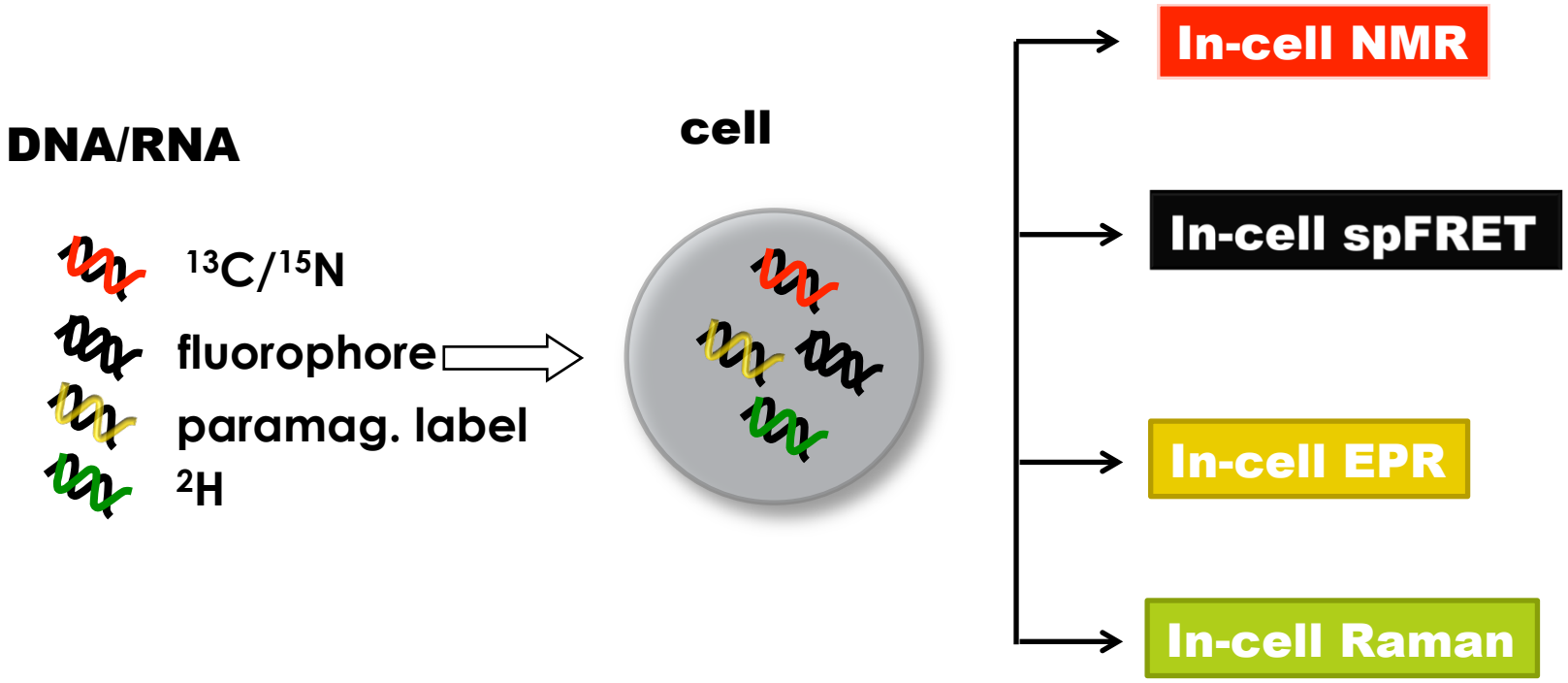
Interepretation based on rigid arrangement of tags might be biased



In-cell A-DNA B-DNA

Nucleic Acids

Structural analysis under in vivo conditions

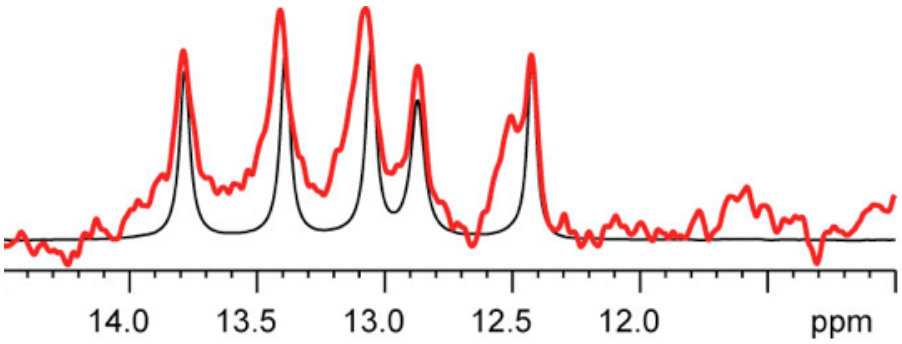


Comparison of in-cell methods

	In-cell NMR	In-cell PELDOR	In-cell spFRET
Disturbance of native environment	Yes	Yes	No
Cell type	*X. laevis* egg/oocyte	*X. laevis* egg/oocyte	*E. coli*, mammalian cells[a]
Toxicity	Sequence dependent[b]	Sequence dependent[b]	No
Subcellular localization	Nucleus/cytosol[c]	Nucleus/cytosol[c]	Nucleus[d]
Tag requirement	No	Yes	Yes
Measurement time span	Hours	< 70 min[e]	Hours
Structural information[f]	Short-range	Long-range	Long-range

in-cell NMR of nucleic acids in mammalian cells

SLO-delivered dsDNA in HeLa cells



(R. Hänsel and V. Dötsch – unpublished)

In-cell Raman microscopy (mammalian cells): under development

^{1}H labeled DNA/RNA